FUNGAL BIOPESTICIDE PRODUCTION BY SOLID-STATE FERMENTATION

GROWTH AND SPORULATION OF *CONIOTHYRIUM MINITANS*

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FUNGAL BIOPESTICIDE PRODUCTION BY SOLID-STATE FERMENTATION

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 15 maart 2000
des namiddags te vier uur in de Aula.

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STELLINGEN:

1. Wanneer stellingen bedoelt zijn om te laten zien dat een promovendus verder gekeken heeft dan zijn of haar neus lang is, is het wellicht zinvoller om in plaats van tenminste 6 wetenschappelijke stellingen tenminste 6 maatschappij-kritische stellingen te eisen.

   Artikel 2.1c en 5.1 promotiereglement Wageningen Universiteit.

2. Wanneer genetische modificatie alleen ingezet mag worden voor 'maatschappelijk verantwoorde' doelen, is niet het bepalen of een doel maatschappelijk verantwoord is het probleem maar het bepalen wie de wijsheid hebben om die beslissing te nemen.

3. Wanneer wetenschappers zelf oproepen (in een open brief dd. 26 september 1999) tot onmiddellijke opschorting van alle maatregelen tot vrijlating van transgene gewassen in het milieu, maken consumenten zich terecht druk om genetische modificatie.

   HN, 23 oktober 1999

4. Wanneer gezondheidsclaims betreffende 'functional foods' gebaseerd moeten zijn op wetenschappelijk onderzoek, is het dubieus dat producenten van deze producten hun onderzoeksactiviteiten m.b.t. deze functionele voedingsmiddelen uitbreiden.

   Food Management 1999, jaargang 17, no. 14, p6.

5. In tegenstelling tot wat veel mensen denken, is wetenschap nooit objectief.

6. *Meten is weten*, desondanks is het opzetten en uitvoeren van goede analysemethoden in de wetenschap een ondergewaardeerd gebied.

7. Het bij consumenten heersende idee dat EKO-, biologische, en natuurlijke producten per definitie gezond zijn, is niet gebaseerd op harde gegevens.

8. De opmerking dat de weerstand van consumenten tegen genetische modificatie zal verdwijnen wanneer zij producten geproefd hebben met gezondheidsbevorderende of verbeterde organoleptische eigenschappen, is te optimistisch.

   German et al (1999), Trends Biotechnol., 17, 492-499; Cantor (2000), Trends Biotechnol., 18, 6-7.

9. Het zou veel onderzoekers een hoop werk besparen wanneer negatieve resultaten net zo makkelijk gepubliceerd zouden kunnen worden als positieve.

10. In de directe synthese van antibiotica met verschillende azijnzuurderivaten als een van de substraten is het gebruik van acetaat als buffercomponent niet geoorloofd.

    Seijas et al (1993) US patent 5268271

11. Door zich ten onrechte op een massabalans van 100% te beroepen komen Svedas et al (1980) tot de conclusie dat directe synthese van semi-synthetische antibiotica mogelijk is. In praktijk blijkt dit echter onmogelijk.

    Svedas et al (1980), Enzyme and Microbiol. Technol., 2, 138-144; Diender et al, (1998), J. Mol. Catal. B-enzyme, 5, 1-4; Schroën et al (1999), Enzyme Microbiol. Technol., 24, 498-506

12. Aangezien Engelkes et al (1997) verschillende stikstofbronconcentraties getest hebben bij maar één koolstofbronconcentratie concluderen zij ten onrechte dat groei, sporulatie en efficiency beïnvloedt wordt door de C/N-ratio.

    Engelkes et al (1997), Phytopathology 87, 500-505

13. In het streven naar beter beheersbare processen en een constante product kwaliteit kan het gebruik van gedefinieerde media in vaste-stoffermentaties uitkomst bieden.

    Hoofdstuk 6 van dit proefschrift

14. Niet steriel bedreven, simpele apparatuur en makkelijke product opwerking zijn veel gehoorde voordelen van vaste-stoffermentaties t.o.v. vloeistof fermentaties die echter lang niet altijd waar zijn.

    o.a. Hoofdstuk 6 van dit proefschrift, Smits et al (1998); Agro-Food-Industry Hi-Tech, 9, 29-36.

Stellingen behorende bij het proefschrift:
Fungal biopesticide production by solid-state fermentation:
Growth and sporulation of *Coniothyrium minitans*

L.P. Ooijkaas
Wageningen, 15 maart 2000

VOORWOORD

Ga uit van je eigen capaciteiten, en je eigen capaciteiten reiken verder dan jezelf vermoed. Deze uitspraak geldt voor veel mensen maar zeker voor mij. Volgens mijzelf was OiO worden niets voor mij, ik was daar niet goed genoeg, niet slim genoeg voor. En ziehier. Voor u ligt mijn proefschrift, het resultaat van 4 jaar OiO-onderzoek. Blijkbaar reiken ook mijn capaciteiten verder dan ik zelf ooit vermoed had.

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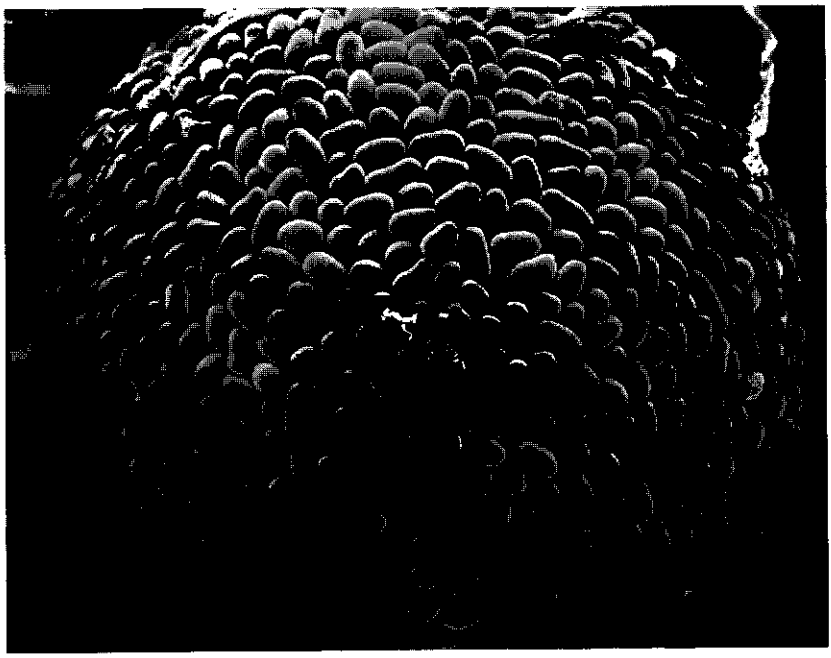
Lydia

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION



CHAPTER 1

BIOLOGICAL CONTROL WITH FUNGI

Chemical pesticides have been used extensively to control insects, weeds and fungal and bacterial diseases because of their effectiveness and ease-of-use. However, more rigorous registration requirements have led to the situation that many of the most effective chemicals have failed or will fail to pass re-registration. The withdrawal of these chemicals coupled to the public opinion towards agrochemicals and the emergence of pesticide-resistant pathogens have created opportunities for biological control agents (Fokkema, 1996; Jackson, 1997).

In literature, a large number of fungi are described which are promising candidates for development as biopesticide. Despite the success in discovering potential biocontrol agents only a few of them are available for commercial use mainly due to the, at least initially, relatively small niche markets and high registration costs (Rodgers, 1993; Fokkema, 1996). Table I gives some fungal biocontrol agents commercially available against insects, weeds and fungal diseases. Some of these products, e.g. Contans, have just recently entered the market and are only registered in one or two countries, while others have been available for quite some years and are already more widely registered, e.g. Rotstop, P.g. suspension, Trichodex and Binab T.

SOLID-STATE FERMENTATION

In addition to market and regulatory issues, commercial success of biopesticides depends on cost-effective production systems. Production methods must yield high concentrations of effective fungal spores or conidia since these are usually more stable as compared to mycelium (Jackson, 1997). In principle, two methods for spore production can be distinguished, i.e. liquid and solid-state fermentation. Solid-state fermentation (SSF) involves the cultivation of microorganisms on moist solid substrates, usually of agricultural origin. SSF is generally the preferred production method since most fungi sporulate well on solid substrates. In addition, SSF produces biocontrol agents of better quality than liquid fermentation (Silman et al, 1993; Desgranges et al, 1993; Muñoz et al, 1995).

Table I: Commercial biocontrol agents based on fungi for use against insects, weeds and (fungal) plant diseases

	Fungus	Target pathogen/disease	Trade name	Manufacturer/distributor
Insects	*Beauveria bassiana*	white fly	Mycotrol	Mycotech Corp[1]
	Metharhizium anisopliae	termites, cockroaches	Bio-blast, Bio-path	EcoScience[1, 2]
	Peacilomyces fumsorosus	white fly	PreFaRal WG	Biobest[2]
	Verticillium lecanii	white fly	Mycogermin, Mycotal	Chr. Hansen[3], Koppert[2, 3]
Weeds	*Colletotrichum gloeosporioides*	*Aeschynomene virginia*	Collego, Biomal	Ecogen Inc[1, 3], Philom Bios[3]
	Phytophthora palmivora	*Morrenia odorata*	Devine	Abott[1, 3]
Diseases	*Ampelomyces quisqualis*	powdery mildew	AQ10 biofungicide	Ecogen Inc[4]
	Candida oleophila	*Botrytis* spp, *Penicillium* spp	Aspire	Ecogen Inc[4]
	Coniothyrium minitans	*Sclerotinia sclerotiorum, S. minor*	Contans	Prophyta[2]
	Gliocladium virens	*Rhizoctania solani, Phytium* spp	Soilgard (Gliogard)	Grace/Sierra[3, 4]
	Gliocladium catenulatum	*Phytium* spp, *Rhizoctania solani*, *Botrytis* spp, *Didymella* spp	Gliomix	Kemira Agro Oy[4]
	Phlebia gigantea	*Heterobasidium annosum*	Rotstop, P.g. Suspension	Kemira Agro Oy[4]
	Phytium oligandrum	*Phytium ultimum*	Polygandron	Vyskumny ustav rastlinnej[4]
	Trichoderma harzianum	*Sclerotinia* spp, *Phytophthora*	Binab T	Bio-Innovation AB (BINAB)[3, 4]
	and other spp	*Rhizoctania solani, Phytium* spp, *Fusarium* spp, *Botrytis cinerea*	Trichodex	Makhteshim Chemical Works[4]
			Trichopel, Trichoject	Agrimm Technologies[4]
	Verticillium dahliae	Dutch elm disease	Dutch Trig	Arcadis (Heidemij)[4]

[1] Jackson et al, 1997, [2] Company's WebPages, [3] Rodgers, 1993, [4] Fokkema, 1996

SSF is a very old technique; it is used from ancient times for the production of fermented foods, especially in the Orient. SSF is now widely used for laboratory evaluation as well as field tests for biocontrol agents. Even some of the commercially available biological control agents of Table I are produced by SSF. However, the design, upscaling and operating rules are still determined by trial and error. Rational design and operation of an SSF process for production of fungal conidia are hampered by several factors. One of them is the lack of knowledge about the physiology and the kinetics of fungal growth and sporulation in SSF.

CONIOTHYRIUM MINITANS

As mentioned in Table I, *C. minitans* is a biocontrol agent of *Sclerotinia sclerotiorum*, a widespread plant pathogen affecting more than 360 plant species. This pathogen survives as sclerotia in the soil and may remain viable for many years. These sclerotia may germinate to produce mycelia to infect plants directly, or to produce apothecia that release ascospores that can infect aerial plant parts (McQuilken and Whipps, 1995). In Figure 1 the life cycle of *S. sclerotiorum* is schematically depicted. The hyphal tips of *C. minitans* are able to penetrate the cell walls of mycelia and sclerotia of *S. sclerotiorum* without the formation of specialized structures. As a result the protoplasm of *S. sclerotiorum* disintegrates and the cell wall collapses; consequently infected sclerotia become soft, disintegrate and fail to germinate (Punithalingam, 1982).

As with other biopesticides, large quantities of effective spores are needed. Since small spore numbers are obtained in liquid cultures while high numbers are produced by SSF, an SSF process is the preferred mass production method for this fungus (McQuilcken et al, 1997; McQuilken and Whipps, 1995).

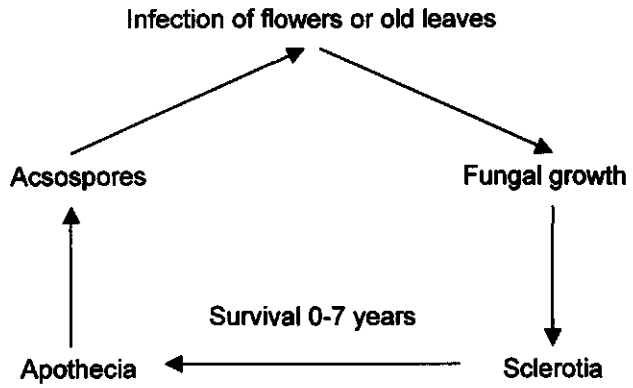
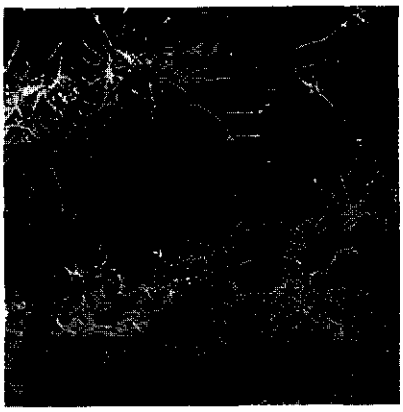


Figure 1: Life cycle of *S. sclerotiorum*. *C. minitans* infect the mycelia and sclerotia, thereby destroying the pathogen infectivity.

C. minitans, a coelomycete, forms spores within bodies lined by fungal tissue, the so-called pycnidia. These pycnidia appear in the mycelia as denser tufts, which become darker with age. The spores arise form the innermost layer of the pycnidia and exude from these pycnidia as a dark-brown to black liquid mass. Figure 2 shows SEM-pictures of pycnidia.

A



B

Figure 2: SEM-pictures of pycnidia of *C. minitans*. Pycnidia laying in chains among hyphe (a) and pycnidia exuding spores (b)

Depending on the substrate, the sizes of pycnidia and spores, colony morphology, and spore production may vary (Whipps and Gerlagh, 1992). However, information on how cultivation conditions and substrate properties affect the growth, pycnidia formation and spore production is sparse.

OUTLINE OF THIS THESIS

In this thesis, research on the physiology and kinetics of growth and sporulation of *C. minitans* in SSF is described. The aim was to determine the optimal substrate composition for spore production and to determine the kinetic parameters and stoichiometry of the bioconversion reactions. The quantity of biomass is an essential variable in characterizing the optimal growth and sporulation conditions. However, direct measurement of biomass is almost impossible in SSF since fungi penetrate into and bind tightly to the solid substrate. Therefore, various indirect methods to estimate the amount of biomass were evaluated (Chapter 2).

The influence of several carbon and nitrogen sources on sporulation using chemically defined media, which facilitate reproducible studies, is described in Chapter 3. The medium giving the best results was optimized with respect to spore quantity (Chapter 4) using statistically based experimental designs. These designs are more efficient than varying one factor at a time.

In Chapter 5, the study on stoichiometry and kinetics of mycelium and spore production is reported. The outcome was used for preliminary cost calculations.

In all these laboratory studies the use of a chemically defined medium was very useful. In industrial processes the use of an inert carrier impregnated with a chemically defined medium as alternative for agricultural substrates in SSF might be attractive too. The potential of such systems, inert carriers impregnated with defined media, is discussed in Chapter 6.

REFERENCES

Desgranges, C., Vergoignan, C., Lérec, A., Riba, G. and Durand, A. 1993. Use of solid-state fermentation to produce *Beauveria bassiana* for the biological control of European corn borer. Biotech. Adv., 11, 577-587.

Fokkema, N.J. 1996. Biological control of fungal plant diseases. Entomophaga, 41, 333-342.

Jackson, M.A. 1997. Optimizing nutritional conditions for the liquid culture production of effective fungal biocontrol agents. J. Ind. Microbio. Biotechnol., 19, 180-187.

McQuilken, M.P., Budge, S.P. and Whipps, J.M. 1997. Production, survival and evaluation of liquid-culture produced inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Biocontr. Sci. Technol., 7, 23-36.

McQuilken, M.P. and Whipps, J.M. 1995. Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Eur. J. Plant. Pathol., 101, 101-110.

Muñoz, G.A., Agosin, E., Cotoras, M., San Martin, R. and Volpe, D. 1995. Comparison of aerial and submerged spore properties for *Trichoderma harzianum*. FEMS Microbio. Lett., 125, 63-70.

Punithalingam, E. 1982. *Coniothyrium minitans*. CMI Descriptions of pathogenic fungi and bacteria no. 732. Commonwealth Mycological Institute, Kew, Surrey, England.

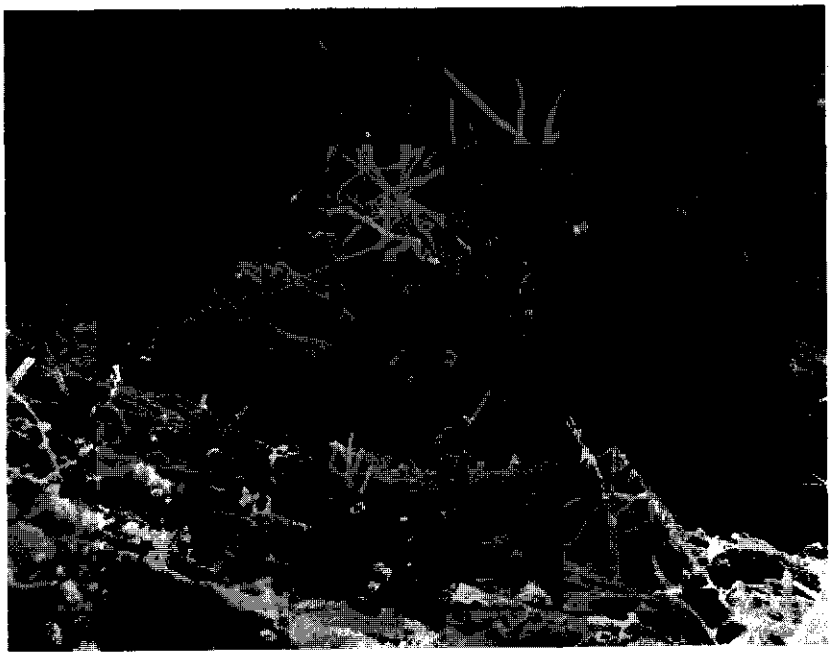
Rodgers, P.B. 1993. Potential of biopesticides in agriculture. Pestic. Sci., 39, 117-129.

Silman, R.W., Bothast, R.J. and Schisler, D.A. 1993. Production of *Colletotrichum truncatum* for use as a mycoherbicide: effects of culture drying and storage on recovery and efficacy. Biotech. Adv., 11, 561-575.

Whipps, J.M. and Gerlagh, M. 1992. Biology of *Coniothyrium minitans* and its potential for use in disease control. Mycol. Res., 96, 897-907.

CHAPTER 2

BIOMASS ESTIMATION OF *CONIOTHYRIUM MINITANS* IN SOLID-STATE FERMENTATION



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CHAPTER 2

ABSTRACT

Various indirect methods are available to estimate the amount of biomass in solid-state fermentation. The suitability of respiration measurements and of measurements of the content of various cell components were evaluated for *Coniothyrium minitans* grown on different media. Protein and nucleic acid measurements are useful as indicators for total biomass when *C. minitans* is grown on potato dextrose agar (PDA) but not when grown on nutrient agar with glucose (NAG) or starch (NAS) as a carbon source. When used alone, none of the methods tested are reliable enough to discriminate between the amounts of mycelial and spore biomass of *C. minitans*. However, a distinction can be made between mycelial and spore biomass on PDA by combining the content of glucosamine, protein and nucleic acid with each other or with spore counts.

INTRODUCTION

In agriculture, chemical pesticides are predominantly used to control pests and diseases. New restrictions on the application of these chemicals and environmental considerations have led to an increased interest in biocontrol agents. A range of biocontrol agents, such as bacteria, fungi and nematodes, is used to control insect pests, fungal and bacterial diseases and weeds (Rodgers, 1993). The fungus *Coniothyrium minitans* is an example of a biocontrol agent. This fungus can be used to control the widespread plant pathogen *Sclerotinia sclerotiorum* that causes severe crop losses during growth and storage of a wide range of plants (Campbell, 1947; McQuilken and Whipps, 1995; Whipps and Gerlagh, 1995). Widespread application of this fungal antagonist depends on the mass production of the spores. Spores of *C. minitans* have only been successfully produced by solid-state fermentation (SSF), i.e. growth of the fungus on moist solid material.

Little is known about the influence of nutritional and environmental factors on the physiology and kinetics of the growth and spores production of *C. minitans*. The quantity of biomass is an

essential parameter in kinetic studies and in characterizing the optimal growth and sporulation conditions for *C. minitans*. In the case of SSF, direct measurement of fungal biomass is hampered because fungi penetrate into and bind themselves tightly to the solid-substrate particles. Many authors have described indirect methods to estimate biomass in SSF. These indirect methods are based on measuring the contents of certain cell components such as chitin (Arima and Uozumi, 1967; Boyle and Kropp, 1992; Desgranges et al, 1991a; Matcham et al, 1985), ergosterol (Desgranges et al, 1991a; Matcham et al, 1985; Nout et al, 1987), protein (Boyle and Kropp, 1992; Córdova-López et al, 1996), and nucleic acid (Arima and Uozumi, 1967; Bajracharya and Mudgett, 1980; Koliander et al, 1984), or measuring the biological activity, e.g. respiration (Carrizalez et al, 1981; Oriol et al, 1988). However, all these indirect methods have their own limitations, which will be illustrated below.

The contents of the different cell components can change markedly in fungi, depending on fungal species, growth conditions and culture age. Furthermore, the length of the analytical procedures, the laborious preparations needed to measure the cell components and the possible interference of (natural) substrate components with the assays can be major drawbacks of these methods. Measuring the oxygen consumption and/or carbon dioxide production (respiration activity) is another method to estimate the amount of biomass (Oriol et al, 1988). This method provides on-line information. However, the cumulative CO_2 production does not always reflect the total accumulated biomass (Larroche et al, 1986).

For these reasons it is not possible to predict which indirect method to estimate fungal biomass in SSF is useful for *C. minitans*. To compare different growth conditions, the contents of the selected indicator must ideally be constant throughout fungal development and must be the same under different cultivation conditions. In this study different methods to estimate biomass are evaluated for *C. minitans*. Agar plates covered with a membrane were used as a model system for SSF. This system prevents penetration of the mycelium into the agar and allows the complete recovery of fungal biomass, so the amount of cell constituents can be related to the biomass dry weight. The content of glucosamine, ergosterol, protein, total carbohydrate and nucleic acids and their changes during cultivation of *C. minitans* on this model system were

determined. The oxygen consumption and carbon dioxide production of this fungus on different media were studied as well.

MATERIALS AND METHODS

Microorganism, inoculum preparation

The microorganism used was *C. minitans* isolate IVT-1 (CBS 14896), kindly provided by Dr. M. Gerlagh from the IPO-DLO Research Institute for Plant Protection, Wageningen, The Netherlands.

The inoculum was produced by cultivation of *C. minitans* on potato dextrose agar (PDA, Difco). The spores were harvested as described below. The stock solution prepared in this way was divided into smaller portions and stored in 20% ($w.v^{-1}$) glycerol at -80 °C.

Media, inoculation and incubation

Three different media were used: PDA (Potato Dextrose Agar, Difco) and nutrient agar (Oxoid) with either 20 $g.l^{-1}$ glucose (NAG) or soluble starch (Merck, NAS) as a carbon source. Petri dishes with 15 ml agar media were covered with a sterilized and pre-weighed nylon membrane filter (Schleicher and Schuell, NY-13-N, 0.45 μm). The Petri dishes were inoculated with 100 μl spore suspension containing 10^4 spores.ml^{-1} which were homogeneously spread over the surface of the membrane filter (45 dishes per medium). The dishes were placed in a desiccator with a volume of approximately 20 l (one desiccator per medium) and incubated at 20 °C. The amount of oxygen and carbon dioxide in the desiccator was determined by GC analysis before and after opening and closing the desiccator to take out three Petri dishes for harvesting the spores and mycelium.

Harvest

The spores were harvested from the surface of the filter by adding 10 ml milliQ water and scraping the surface with a bent glass rod. The suspension was filtered through two layers of

cheesecloth to separate the mycelium (stays behind) and the spores (pass through the cheesecloth). The mycelial residue was replaced on the membrane filter used for cultivation and the procedure was repeated until the filtrate was clear (at least two times). Then the residue was placed on the membrane filter again and dried in an oven at 60°C for 48 h.

The filtrates were combined and adjusted to a known volume. The spores in the combined filtrate were counted using a Neubauer counting chamber. After being counted, the suspension was vacuum filtered through a pre-weighed filter (Schleicher and Schuell, ME-24, 0.2 μm) and the filter was dried in an oven at 60 °C for 48 h.

For dry weight measurements the filters were re-weighed after being cooled in a desiccator. The dried mycelia or spores were scraped from the filters. After being weighed, material from the triplicate samples was combined to have sufficient material for the analysis of the cell components.

Oxygen and carbon dioxide

Gas analysis was carried out by injecting the gas in a micro gas chromatograph (Chrompack, CP-2002) fitted with an automatic sampling port and a thermal conductivity detector. A HayeSep A column (Chrompack) was used for CO_2 determination and a Molsieve 5Å PLOT column (Chrompack) for O_2 determination.

Biochemical analyses

The contents of the different cell components were analyzed in separated and dried mycelium and dried spores. The different components were extracted from the dried material following the sequential extraction given in Figure 1.

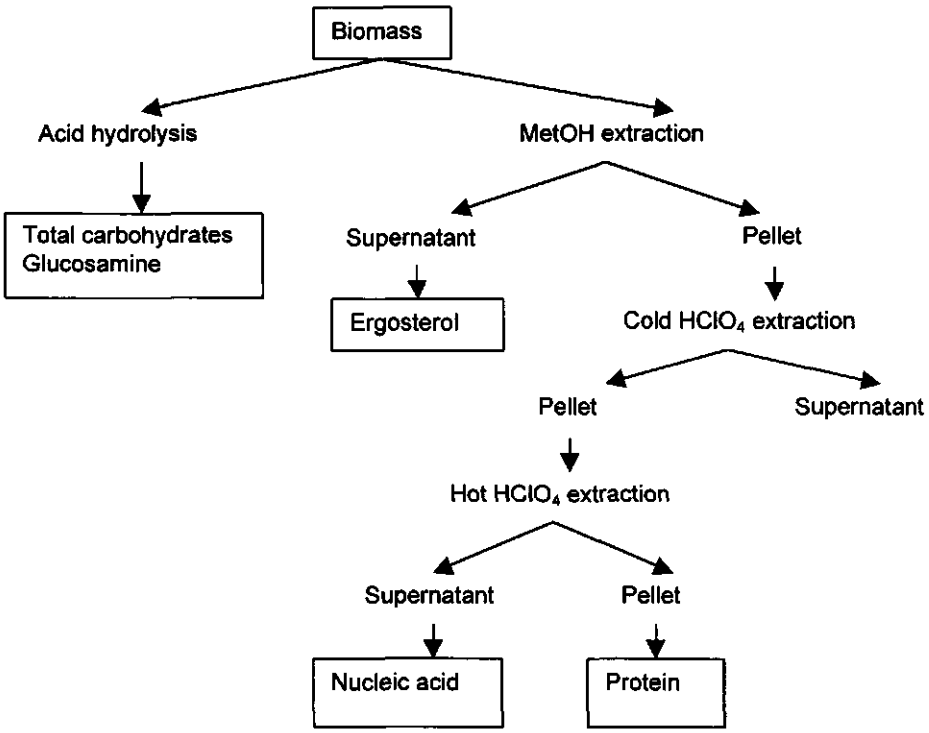

Figure 1: Sequential extraction procedure for the analysis of the various cell components

Total carbohydrate and glucosamine

Total carbohydrates and glucosamine were determined after an acid hydrolysis. To 5 mg dry sample 5 ml of 6 M HCl were added in glass tubes with Teflon screw caps and the tubes were incubated at 100 °C for 4 h. After this hydrolysis step the HCl was evaporated under vacuum at 45 °C in a rotavapor. The dry residue was dissolved in 5 ml milliQ water (sample solution). Total carbohydrates were measured with the Anthrone-method (Herbert et al, 1971). To one ml of sample solution 5 ml of Anthrone reagent were added. This Anthrone reagent was composed of 200 mg Anthrone (Merck) and 5 ml absolute ethanol which was made up to 100 ml with 75% H_2SO_4. The solution was mixed and incubated at 100 °C for 10 min. After the solution had been cooled, the absorbance was read at 625 nm. Standard solutions of glucose were tested with each assay for quantification purposes.

Glucosamine was assayed on an HPLC by injecting 20 μl of sample solution after filtration through 0.45 μm. The components were separated on the CarboPac1 column (Dionex, 4*250 mm) protected by a Carbopac1 guard column under isocratic conditions with 16 mM NaOH as the eluent at a flow rate of 0.8 ml.min^{-1} and a column temperature of 40 °C. Peaks were detected with a pulsed amperometric detector (Dionex). After each run the column was regenerated by washing it for 10 min with 200 mM NaOH. Standard solutions of glucosamine, galactosamine, glucose, galactose and mannitol were also injected.

Ergosterol

To extract ergosterol 25 mg dry sample in 25 ml methanol was homogenized on ice for 2 min with an Ultra Turrax. The tip of the Ultra Turrax was washed with 5 ml methanol into the homogenized sample. The homogenate was centrifuged (5 min, 1500*g) and the pellet was stored for further analysis. To the supernatant 5 ml 4% (w.v^{-1}) KOH in 95% (v.v^{-1}) ethanol were added. This mixture was saponified for 1 h at 60 °C. After this mixture had been cooled to room temperature 10 ml of distilled water were added. A series of three petroleum ether (bp 40-60 °C) extractions (10, 5 and 5 ml) was performed. The petroleum ether fractions were combined and evaporated under vacuum at 30 °C in a rotavapor. The dried residue was dissolved in 3.0 ml methanol.

Ergosterol was measured by HPLC analysis of the filtered sample (Nuclepore, PTFE, 0.45 μm). The sample was injected on a Sephasil C18 column (Pharmacia, 4*250 mm) protected by a Sephasil C18 guard column. Ergosterol was eluted with 97.5 % (v.v^{-1}) methanol at a flow rate of 1.0 ml/min and a column temperature of 40 °C. Peaks were detected with an UV-detector (Waters) at a wavelength of 282 nm. A standard solution of ergosterol (Sigma) in methanol was used for quantification purposes.

Nucleic acid

To the pellet of the ergosterol extraction 5.0 ml 0.25 M cold perchloric acid ($HClO_4$) were added. This mixture was incubated 30 min at 4 °C. After having been centrifuged (5 min, 10000*g) the supernatant was discarded. To this pellet 4.0 ml 0.5 M $HClO_4$ were added. This

mixture was incubated for 15 min at 70 °C. After centrifugation (5 min, 10000*g) this procedure was repeated twice with 3 ml of 0.5 M $HClO_4$. The supernatants were combined and the absorbance at 260 nm was read. The concentration of nucleic acids in the supernatant was calculated on the assumption that a solution containing 1 $mg.ml^{-1}$ nucleic acids has an absorbance of 23 at 260 nm (Hanson and Phillips, 1981).

Protein

To the pellet obtained after the nucleic acid analysis 1.0 ml 1.0 N NaOH was added. After incubation at 95 °C for 5 min the mixture was centrifuged (5 min, 10000*g). The protein in the supernatant was measured using the commercial BCA Protein Assay of Pierce (Pierce). To 0.1 ml sample 2.0 ml BCA reagent (Pierce) were added. After incubation at 60 °C for 30 min and cooling down to room temperature the absorbance at 562 nm was read. Standard solutions of bovine serum albumin (Pierce) were included in each assay.

Replication and statistical treatment of results

Measurements of dry weight and spore numbers were made on three different Petri dishes per medium. Analysis of gas composition was carried out four times before and four times after opening and closing the desiccator. The contents of the various cell components were determined in duplicate. Data of the biochemical analyses were subjected to analysis of variance (ANOVA). The error mean square was obtained from the analysis of variance and used to calculate the least significant difference (LSD, P=0.05). Variations between data are significant when the difference between data is more than the LSD.

The data presented here are means of triplicate (dry weights and spore number) or duplicate (biochemical analyses) samples from one single experiment which is representative of experiments previously performed.

RESULTS AND DISCUSSION

Growth curves

The growth curves of *C. minitans* on different media are shown in Figures 2a, b and c. Spore production starts two or three days later than mycelium growth. On PDA the amount of spores produced is higher than on NAG or NAS both in number ($2.3*10^9$ spores.dish^{-1}, vs. $0.5*10^9$ and $1*10^9$ spores.dish^{-1}) and in weight (65 mg.dish^{-1}, vs. 45 and 50 mg.dish^{-1}).

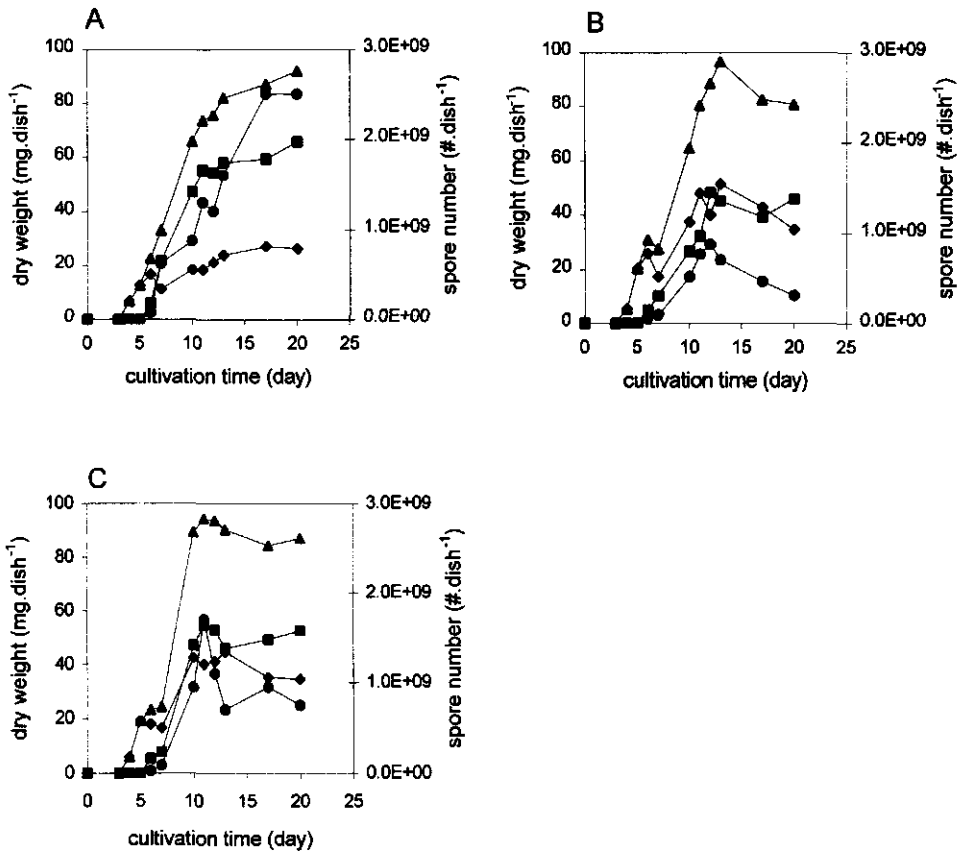

Figure 2: Time courses of biomass production on PDA (a), NAG (b) and NAS (c) of *C. minitans*. Mycelium (◆), spores (■) total biomass (▲) and number of spores (●).

At the end of the growth period the ratios between mycelium and total dry weight and the ratios between spores and total dry weight are different for the different media which is presented in Table I. As can be seen from Table I, the highest ratio between spore and total dry weight is obtained on PDA.

Table I: Final amounts of total, mycelial and spore dry weight, CO_2 produced and O_2 consumed on the media tested at the end of the cultivation time (day 20).

	Media		
	PDA	NAG	NAS
Total dry weight (mg.dish^{-1})	92	81	87
Mycelial dry weight (mg.dish^{-1})	26	35	35
Spore dry weight (mg.dish^{-1})	66	46	52
CO_2 (mmoles.dish^{-1})	5	7	9
O_2 (mmoles.dish^{-1})	4.5	6.5	8

These results suggest that PDA is more efficient for spore production than NA. A possible explanation for these differences in results on PDA and NA could be that the nutrient composition and pH are not the same for the three media. Which factor causes the difference in spore production is not yet known, but will be investigated further.

These three spore production media were used to evaluate different indirect methods, i.e. respiration measurements and biochemical analysis, to estimate the amount of biomass in SSF for *C. minitans*.

Oxygen and carbon dioxide measurements

Figure 3 shows the results of the total amount of O_2 consumed and CO_2 produced on PDA, NAG and NAS. As can be seen, the time courses of O_2 consumption and CO_2 production are similar for all three media. There is a strong increase in O_2 consumption and CO_2 production in the period between 6-7 days till 12-14 days. After this period the amount of O_2 consumed and CO_2 produced only slightly increases.

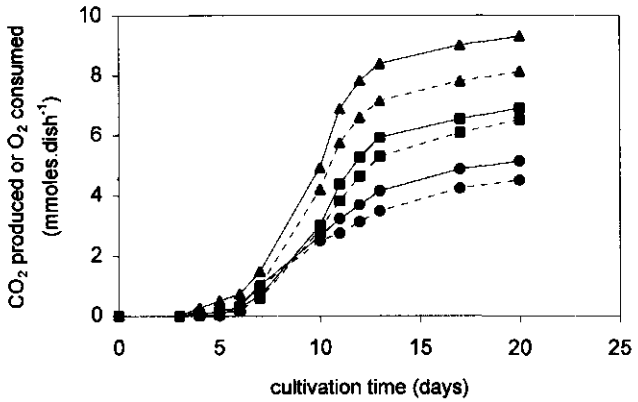

Figure 3: Time courses of O_2 consumption (dashed lines) and CO_2 production (solid lines) on PDA (●), NAG (■) and NAS (▲).

The final amount of CO_2 produced, final amount of O_2 consumed and the final amounts of total biomass, mycelium and spores produced are given in Table I. It can be seen that the total CO_2 production or O_2 consumption does not reflect the mycelium, spores or total biomass production. Figures 4a, b and c show the correlations between CO_2 production and biomass production for the three media. For O_2 consumption similar figures are obtained (results not shown).

Based on these results we conclude that CO_2 production and O_2 consumption (respiration measurements) are less suitable as indicators for biomass in this case. When CO_2 production and O_2 consumption are completely growth-associated, the amount of biomass produced per unit of gas metabolized should be constant in time. However, as can be seen in Figure 4, this is not the case for *C. minitans*. Similar results are obtained for *Aspergillus oryzae* grown on rice where the ratio of CO_2 produced to mycelium formed increased in time (Sugami and Okazami, 1979). On the contrary, using glucosamine as an indicator for biomass of *Beauveria bassiana*, Desgranges and co-workers (1991b) found that the amount of biomass produced per unit of gas metabolized is constant over time. However, this ratio was not the same for the different media. Okazaki and co-workers (1980) suggest that CO_2 production and O_2 consumption are

associated with both maintenance and growth. It is unclear if by growth they mean mycelium production only or mycelium and spore production together. In our case with *C. minitans* mycelium is not only formed but spores are produced too. It is not yet known how maintenance, mycelial growth and spore production of *C. minitans* correlates with O_2 consumption and CO_2 production. Although CO_2 or O_2 measurements are not perfect as indicators for the amount of biomass produced, following the CO_2 production and/or O_2 consumption during the cultivation period gives valuable information on the progress of the spore production process on the media tested.

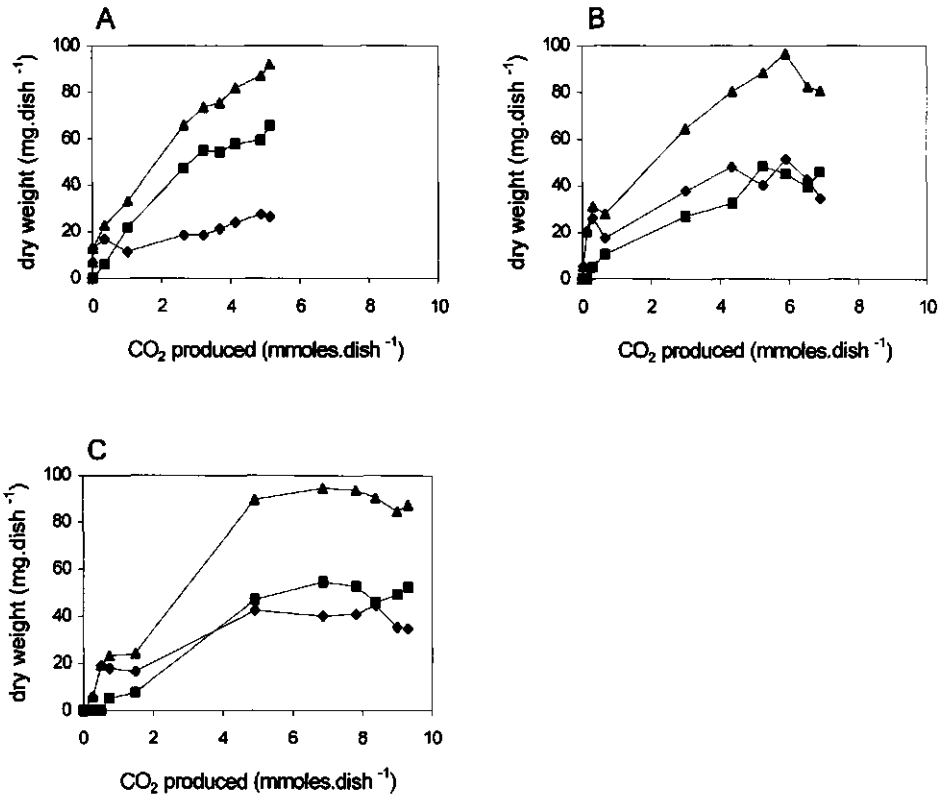

Figure 4: Correlations between CO_2 production and biomass production on PDA (a), NAG (b) and NAS (c). Total biomass (▲), mycelium (◆) and spores (■).

Biochemical analyses

The amount of biomass can also be estimated from measurements of a specific component. The content of this component in relation to the dry weight should then be constant over time. The results of the biochemical analyses are presented in Figures 5-9.

Glucosamine

From Figure 5 it can be seen that the glucosamine content of mycelium on NA increases during the cultivation period (15-25%) while on PDA, after day 7, the content is constant (15%, except for day 13). There is no significant difference in content of the mycelium between NAG and NAS.

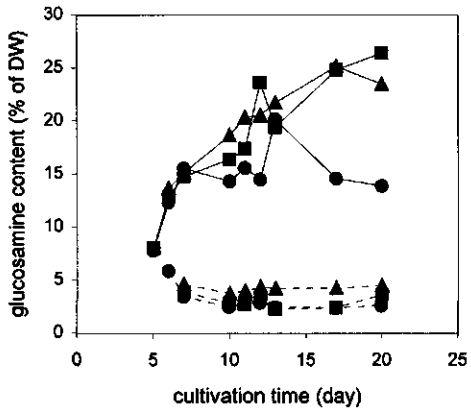

Figure 5: Content of glucosamine expressed as % of dry weight of mycelium (solid lines) or spores (dashed lines) on PDA (●), NAG (■) and NAS (▲). The least significant difference (LSD) between each point is 1.19%.

The glucosamine content of the spores is constant after 6 days of cultivation for all three media. It appears that spores produced on NAS contain significantly (P=0.05) more glucosamine than spores derived from PDA and NAG (average content: 4.2% vs. 2.7% and 3.0%). The

glucosamine content of mycelium is 3 to 5 times higher than the glucosamine content of the spores for all the media tested.

As can be concluded from Figure 5, glucosamine measurements are only useful for biomass estimations after 7 days of cultivation using PDA as medium. More measurements besides glucosamine measurements are necessary to estimate the total amount of biomass because of the difference in glucosamine content between mycelium and spores. In addition, glucosamine measurements alone do not give enough information to distinguish between mycelial biomass and spore biomass. For NA glucosamine measurements are less suitable as a biomass indicator because the content of mycelium increases during fungal development. This increase is probably caused by the resistance of chitin (of which glucosamine is the monomer unit) to breakdown after fungal death (Newell, 1992). Therefore, the chitin can accumulate in the empty ghost hyphae and consequently the glucosamine content of mycelium increases. This increase in glucosamine content is also reported for other fungi, for example *Fusarium oxysporum* (Whipps and Lewis, 1980), *Mycospaerella pinodes* (Ride and Drysdale, 1972), and *A. oryzae* (Arima and Uozumi, 1967). On the contrary, the glucosamine content of *B. bassiana* is constant during the cultivation period (Desgranges et al, 1991a). The difference in results between NA and PDA indicates that the suitability of glucosamine measurements for estimating the biomass for other media should be checked beforehand.

Total carbohydrate

As shown in Figure 6, the carbohydrate content of mycelium on PDA and NAG varies over time while on NAS the content is about constant. In addition, mycelium derived from PDA contains on average more carbohydrates (9.7%) than mycelium derived from NAG (6.9%) or NAS (7.7%). On all three media the total carbohydrate content of spores increases over time but the increase is less on PDA. Furthermore, mycelium contains more carbohydrates than spores for all three media. Therefore, the conclusion is that the total carbohydrate content is unsuitable as indicator for biomass of *C. minitans*. On the contrary, however, the total carbohydrate content of *B. bassiana* is constant over time once sporulation has started

(Desgranges et al, 1991a). However this content varies between media, which is the case for *C. minitans* too.

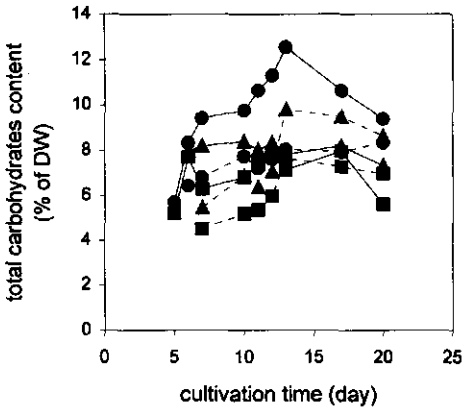

Figure 6: Content of total carbohydrates expressed as % of dry weight of mycelium (solid lines) or spores (dashed lines) on PDA (●), NAG (■) and NAS (▲). The least significant difference (LSD) between each point is 0.95%.

Protein

As shown in Figure 7, the protein content of mycelium decreases to reach a constant value after 10 days. The average content (after 10 days) of mycelium on NAG (3.6%) is comparable with the content on PDA (3.2%), but significantly lower than on NAS (6.0%). The protein content of spores produced on NAG and NAS is higher than on PDA, and decreases in time (14-7% and 26-11%) while on PDA the content is almost constant (5.5%) after 10 days. Mycelium contains less protein than spores for all media, but the difference in content between mycelium and spores is more pronounced on NA than on PDA.

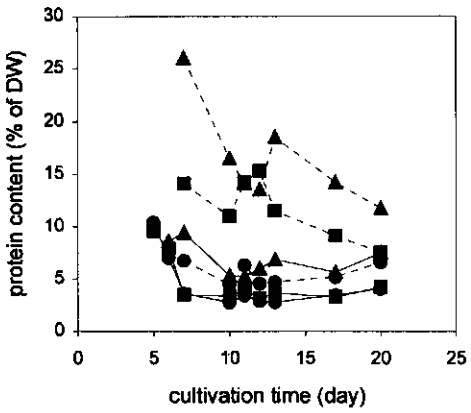

Figure 7: Protein content expressed as % of dry weight of mycelium (solid lines) or spores (dashed lines) on PDA (●), NAG (■) and NAS (▲). The least significant difference (LSD) between each point is 2%.

Protein measurements can thus only be used as an indicator for biomass in the stationary phase, but not in the active growth phase using PDA as a medium. The small but significant difference in protein content between mycelium and spores allows a rough estimation of the total amount of biomass produced on PDA. To distinguish between mycelial and spores biomass, other measurements besides protein acid measurements are needed. Protein measurements are not useful as biomass indicator on NAG or NAS because of the decrease in protein content of the spores. The results shown in Figure 7 agree with the results of Boyle and Kropp (1992). They investigated the use of protein for biomass estimations of four fungal species. They also found that the content is not constant over time and is not the same for the different growth media. Córdova-López and co-workers (1996) used the protein content of a 5-day-old culture for biomass estimations of *A. niger*. There was no information about whether this content was constant during the cultivation period.

Nucleic acid

As shown in Figure 8 the nucleic acid content of mycelium derived from PDA shows the same trend as the protein content. The content decreases to reach a constant value after 10 days (0.9%). For NAG and NAS the content slightly decreases over time which make nucleic acid content unsuitable as biomass indicator on these media

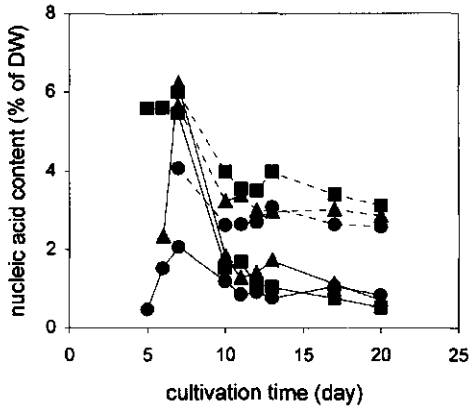

Figure 8: Nucleic acid content expressed as % of dry weight of mycelium (solid lines) or spores (dashed lines) on PDA (●), NAG (■) and NAS (▲). The least significant difference (LSD) between each point is 0.28%.

The nucleic acid content of spores shows the same pattern as the content of mycelium: after day 10 the content of spores obtained from PDA (2.7%) or NAS (3.0%) is constant. These results for nucleic acid content of *C. minitans* are similar to results obtained for *A. oryzae* grown on rice (Arima and Uozumi, 1967; Bajracharya and Mudgett, 1980). During early growth the nucleic acid content is higher and the content levels off in the late growth phase for both *A. oryzae* and *C. minitans*. However, Bajracharya and Mudgett (1980) calibrated the method in submerged cultures, and the question is whether the nucleic acid content in SSF is the same as the content in submerged fermentation.

Ergosterol

The changes in ergosterol content are presented in Figure 9. As can be seen, the ergosterol content of mycelium varies considerably (between 0.1-0.4%); the ergosterol content of the spores produced on NAS shows more variation than that of the other media. In addition, spores produced on PDA contain significantly less ergosterol (0.1%) than spores produced on NA (0.4%). Furthermore, mycelium obtained from PDA contains more ergosterol than spores while on NAG and NAS just the opposite occurs. Due to this variation over time and between media, ergosterol measurements are inapplicable to estimate the amount of biomass. These results for *C. minitans* agree with results found for *B. bassiana*; the ergosterol content of *B. bassiana* varied too throughout time and between media (Desgranges et al, 1991a).

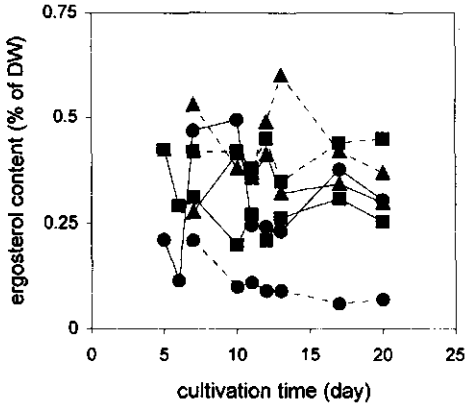

Figure 9: Ergosterol content expressed as % of dry weight of mycelium (solid lines) or spores (dashed lines) on PDA (●), NAG (■) and NAS (▲). The least significant difference (LSD) between is 0.019%.

CONCLUSION

As discussed above, none of the tested indicators are perfect for biomass estimations of *C. minitans* in every situation. However, following the CO_2 production and/or O_2 consumption during the cultivation period gives valuable online information about the progress of the spore

production process on the media tested. Of the biochemical analyses tested only the content of protein or nucleic acid can be used to estimate the amount of total biomass on PDA. Combinations of glucosamine, protein or nucleic acid measurements with each other or with spore counts make it possible to distinguish between mycelial and spore biomass of *C. minitans* grown on PDA in the stationary phase.

Because of the differences in results between PDA, NAG and NAS, it is not possible to predict which indicator is useful for *C. minitans* when other growth media are used. Therefore, it can be concluded that there is no universally applicable indirect method to estimate the amount of biomass of *C. minitans* in SSF. A suitable indirect method to estimate the amount of biomass should be chosen after a custom calibration of the indirect method when other media or other fungal species are used.

ACKNOWLEDGEMENTS

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REFERENCES

Arima, K. and Uozumi T. 1967. A new method for estimation of the mycelial weight in Koji. Agric. Biol. Chem., 31, 119-123.

Bajracharya, R. and Mudgett, R.E. 1980. Effects of controlled gas environments in solid-substrate fermentations of rice. Biotechnol. Bioeng., 22, 2219-2235.

Boyle, C.D. and Kropp, B.R. 1992. Development and comparison of methods for measuring growth of filamentous fungi on wood. Can. J. Microbiol., 38, 1053-1060.

Campbell, W.A. 1947. A new species of *Coniothyrium* parasitic on sclerotia. Mycologia, 39, 190-195.

Carrizalez, V., Rodríguez, H. and Sardiña, I. 1981. Determination of the specific growth of molds on semi-solid cultures. Biotechnol. Bioeng., 23, 321-333.

Córdova-López, J., Gutiérres-Rojas, M., Huerta, S., Saucedo-Castañeda, G. and Favela-Torres, E. 1996. Biomass estimation of *Aspergillus niger* growing on real and model supports in solid-state fermentation. Biotechnol. Techn., 10, 1-6.

Desgranges, C., Vergoignan, C., Georges, M. and Durand, A. 1991a. Biomass estimation in solid-state fermentation. I. Manual biochemical methods. Appl. Microbiol. Biotechnol., 35, 200-205.

Desgranges, C., Vergoignan, C., Georges, M. and Durand, A. 1991b. Biomass estimation in solid-state fermentation. II. On-line measurements. Appl. Microbiol. Biotechnol., 35, 206-209.

Hanson, R.S. and Phillips, J.A. 1981. Chemical composition. In: *Manual of methods for general bacteriology* (Gerhardt, P., Murray, R.G.E. and Costilow, R.N. eds.), American Society for Microbiology, Washington, 328-363.

Herbert, D., Phillips, P.J. and Strange, R.E. 1971. Chemical analysis of microbial cells. In: *Methods in microbiology*, vol. 5b (Norris, J.R. and. Ribbons, D.W., eds.), Academic Press, London, 209-343.

Koliander, B., Hampel, W. and Roehr, M. 1984, Indirect estimation of biomass by rapid ribonucleic acid determination. Appl. Microbiol. Biotechnol., 19, 272-276.

Larroche, C., Desfarges, C. and Gros, J.B. 1986. Spore production of *Penicillium roqueforti* by simulated solid-state fermentation. Biotechnol. Lett., 8, 453-456.

Matcham, S.E., Jordan, B.R. and Wood, D.A. 1985. Estimation of fungal biomass in a solid substrate by three indepent methods. Appl. Microbiol. Biotechnol., 21, 108-112.

McQuilken, M.P. and Whipps, J.M. 1995. Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Eur. J. Plant. Pathol., 101, 101-110.

Newell, S.Y. 1992. Estimating fungal biomass and productivity in decomposing litter. In: *The fungal community, its organization and role in the ecosystem*, 2nd ed. (Caroll, G.C. and Wicklow, D.T. eds.), Marcel Dekker Inc., New York, 521-561.

Nout, M.J.R., Bonants-van Laarhoven, T.M.G., de Jongh, P. and de Koster, P.G. 1987. Ergosterol content of *Rhizopus oligosporus* NRRL 5905 grown in liquid and solid substrates. Appl. Microbiol. Biotechnol., 2, 456-451.

Okazaki, N., Suguma, S. and Tanaka, T. 1980. Mathematical model for surface culture of Koji mold. J. Ferment.Technol., 58, 471-476.

Oriol, E., Schetino, B., Viniegra-Gonzalez, G. and Raimbault M. 1988. Solid-state culture of *Aspergillus niger* on support. J. Ferment. Technol., 66, 57-62.

Whipps, J.M. and Gerlagh, M. 1992. Biology of *Coniothyrium minitans* and its potential for use in disease control. Mycol. Res., 96, 897-907.

Ride, J.P. and Drysdale, R.B. 1972. A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Plant Pathol., 2, 7-15.

Rodgers, P.B. 1993. Potential of biopesticides in agriculture. Pestic. Science, 39, 117-129.

Sugami, S. and Okazami, N. 1979. Growth estimation of *Aspergillus oryzae* cultured on solid media. J. Ferment. Technol., 57, 408-412.

Whipps, J.M. and Lewis, D.H. 1980. Methodology of a chitin assay. Trans. Brit. Mycol. Soc., 74, 417-418.

CHAPTER 3

SPORE PRODUCTION OF *CONIOTHYRIUM MINITANS* DURING SOLID-STATE FERMENTATION ON DIFFERENT NITROGEN SOURCES WITH GLUCOSE OR STARCH AS CARBON SOURCE

This chapter was published in slightly modified form as:

Ooijkaas, L.P., Chin-Joe, I., Tramper, J. and Buitelaar, R.M. 1998. Spore production of *Coniothyrium minitans* on different nitrogen sources with glucose or starch as carbon source. Biotechnology Letters, 20, 785-788.

CHAPTER 3

ABSTRACT

The influence of several nitrogen sources in combination with glucose or starch in low and high concentration on sporulation of *Coniothyrium minitans* was evaluated. Nitrate and histidine influence sporulation negatively at low and high concentrations of both glucose and starch. It was shown that urea and glycine were the preferred nitrogen sources. With these nitrogen sources the pH during cultivation remained stable and high concentrations were not inhibiting when these nitrogen sources were used in combination with starch. Starch was preferred to glucose since spore numbers at high starch concentrations were all a factor two or more higher than at high glucose concentrations.

INTRODUCTION

The fungus *Coniothyrium minitans* is a promising biological control agent of *Sclerotinia sclerotiorum*, a widespread plant pathogen (McQuilken and Whipps, 1995). Commercial application of this biocontrol agent depends on production of large quantities of spores, which requires reliable mass production systems. Since the volumetric yield is higher in solid-state fermentation than in liquid fermentation, a solid-state fermentation process seems to be the preferred mass production method (McQuilken et al, 1997a, b; McQuilken and Whipps, 1995; Ooijkaas et al, 1998). However, a lack of knowledge about the physiology and kinetics of growth and sporulation of *C. minitans* hamper rational design of an industrial solid-state fermentation process.

The un- or semi-defined nature of the media used in the studies mentioned before makes it difficult to investigate the influence of nutritional composition in more detail. To overcome this problem, an inert support impregnated with a defined nutrient solution can be used which will facilitate reproducible spore production and physiological and kinetic studies. Therefore, a chemically defined medium that allows growth and sporulation of *C. minitans* was developed.

Using this defined medium, we studied the influence of the main medium components, carbon and nitrogen, in more detail.

Carbon can be provided in monomeric form, e.g. glucose, which is immediately available for the fungus, or in polymeric form, e.g. starch, which first has to be hydrolyzed by the fungus. Since high glucose concentrations might inhibit growth and/or sporulation, as is the case for *Colletotrichum truncatum* (Jackson and Bothast, 1990), *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus* (Hallsworth and Magan, 1994), starch might be preferred to glucose to use at high concentrations.

Various reports deal with the influence of nitrogen source on growth and sporulation of several fungi (Jackson and Bothast, 1990; Zhou et al, 1996; Engelkes et al, 1997). However, the influence of nitrogen source on sporulation seems to be fungus-specific. The same nitrogen source stimulating sporulation of one species may inhibit sporulation of another species, for example ammonium sulfate stimulates sporulation of *Fusarium graminearum* (Larroche, 1996), but inhibits sporulation of *C. truncatum* (Jackson and Bothast, 1990). Therefore, the effect of nitrogen source in combination with glucose or starch on sporulation of *C. minitans* was investigated.

MATERIALS AND METHODS

Microorganism, media, inoculation and incubation

C. minitans CBS 14896 was grown on a medium which contained the following components: KH_2PO_4 2.0 $g.l^{-1}$, thiamin 2 $mg.l^{-1}$, trace elements stock solution 10 ml/l, Mes (2-[n-morpholino]ethanesulfonic acid) 150 mM, agar 15 $g.l^{-1}$ and glucose or starch combined with NH_4NO_3, $(NH_4)_2SO_4$ + urea, urea, $(NH_4)_2SO_4$, $NaNO_3$, glycine or histidine at an initial C/N ratio ($mol.mol^{-1}$) of 10. In case of $(NH_4)_2SO_4$ combined with urea, both nitrogen sources provide equal amounts of nitrogen on a molar base. The concentrations tested in this study are given in Table I.

Table I: Concentrations tested for the various carbon and nitrogen sources

Component	low concentration ($g.l^{-1}$)	high concentration ($g.l^{-1}$)
Glucose	20.00	100.00
Starch	18.00	90.00
Ammonium nitrate	2.67	13.35
Ammonium sulphate + urea	2.20 + 1.00	11.00 + 5.00
Urea	2.00	10.00
Ammonium sulphate	4.40	22.00
Sodium nitrate	5.66	28.30
Glycine	5.00	25.0
Histidine	3.45	17.25

The trace elements stock solution consisted of (in $g.l^{-1}$): EDTA 1, $CaCl_2.2H_2O$ 0.1, $MgCl_2.6H_2O$ 10, $ZnSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.5, $Na_2MoO_4.2H_2O$ 0.02, $CuSO_4.5H_2O$ 0.02, $CoCl_2.6H_2O$ 0.04 and $MnCl_2.4H_2O$ 0.1.

Petri dishes (9 cm diameter) with 20 ml media were inoculated with 100 μl spore stock suspension ($5*10^5$ spores.ml^{-1}) which was homogeneously spread over the agar surface. The dishes were incubated at 20 °C. To protect the plates against drying out plastic boxes filled with water were placed in the incubator to maintain humidity inside the incubator.

Analysis

All analyses were done after the whole content of each Petri dish had been blended for 1 minute with 40 ml demineralized water. The number of spores in each sample was determined using a Neubauer counting chamber and the pH of each sample was measured. Residual glucose was determined using the enzymatic glucose-oxidase peroxidase assay (Merck-Biotrol) according to the manufacturer's protocol. Starch was determined as the total amount of glucose after an enzymatic hydrolysis with amyloglucosidase. Therefore, 100 μl sample was incubated with 900 μl citric acid buffer (50 mM, pH 4.8) and 0.3 mg amyloglucosidase (75 $U.mg^{-1}$, Merck) for 2 h at 60 °C.

Free glucose liberated from starch by fungal hydrolysis, was measured before this enzymatic hydrolysis. All data here presented are the means of two duplicate experiments. Spore numbers

were subjected to analysis of variance (ANOVA) after log transformation using the Genstat program. Treatment means were compared with the least significant difference (LSD) at a probability of 5%.

RESULTS AND DISCUSSION

Several nitrogen sources in combination with glucose or starch were evaluated for their influence on sporulation of *C. minitans*. Since not only source but also concentration and C/N ratio can affect sporulation, we tested two C-concentrations while keeping the initial C/N ratio constant at 10 (mol.mol^{-1}) by likewise changing the N-concentration. The effect of various nitrogen sources on sporulation is illustrated in Figure 1a and b.

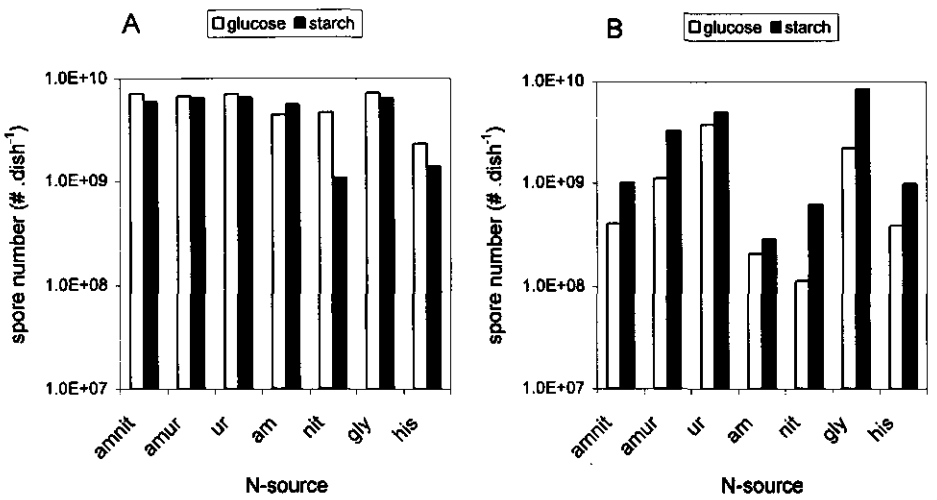

Figure 1: Spore production after 31 days of cultivation on the different nitrogen sources ammonium nitrate (amnit), ammonium sulfate and urea (amur), urea (ur), ammonium sulfate (am), nitrate (nit), glycine (gly) or histidine (his) combined with glucose or starch in low (a) or high (b) concentrations at an initial C/N ratio (mol.mol^{-1}) of 10.

As can be seen in Figure 1a, the nitrogen sources nitrate or histidine gave significant lower spore numbers (1-4*10^9 spores.dish^{-1}) as compared to the other nitrogen sources (5-6*10^9 spores.dish^{-1}).

Possibly not enough energy is available to synthesize a large number of spores when nitrate is used as nitrogen source. Nitrate assimilation is a two-stage process, nitrate being reduced to nitrite and nitrite being reduced to ammonia which requires considerable energy (Jennings, 1995). Similarly, the energy yielded by histidine-use might be not enough either for synthesis of a large number of spores. Breakdown of histidine goes via α-ketoglutarate, a citric-acid-cycle intermediate while breakdown of glycine goes via pyruvate. The latter enters the citric-acid-cycle as acetyl CoA thereby generating more energy than α-ketoglutarate (Stryer, 1975). Hence, the spore numbers obtained with histidine are lower than with glycine (Figure 1a).

When high substrate concentrations were used (Figure 1b), ammonium sulfate and ammonium nitrate, besides sodium nitrate and histidine, gave lower spore numbers (<1*10^9 spores.dish^{-1}) than ammonium and urea, urea or glycine (2-6*10^9 spores.dish^{-1}). Sodium nitrate and histidine result in lower spore numbers for the reason discussed before. The drop in pH from 6.5 to 3 caused by the release of a proton into the medium when the microorganism uses ammonium might explain the results with ammonium sulfate and ammonium nitrate. Since McQuilken et al (1997a) have shown that sporulation of *C. minitans* is inhibited at low initial pH-values (below pH 4.5), it is likely that this acidification caused by high concentrations of ammonium ions results in lower spore numbers. In case of ammonium sulfate combined with urea, the pH drops first from 6.5 to 4 when ammonium is used and raises again to pH 6.5. In case of urea or glycine, the pH remains stable during cultivation. Consequently, higher spore numbers are obtained with these 3 nitrogen sources as compared to ammonium and ammonium nitrate at high concentrations (Figure 1b).

Comparing the spore numbers of glucose with starch, we see that spore numbers at high starch concentrations are a factor two or more higher than at high glucose concentrations. High glucose concentrations are more inhibiting than high starch concentrations, possibly caused by the hydrolysis of starch to glucose. If fungal hydrolysis of starch is slow compared to the glucose uptake rate, the glucose concentration remains low during the whole cultivation period

thereby reducing the negative effect of substrate inhibition by glucose. Residual and free glucose measurements during cultivation of *C. minitans* support this hypothesis. With high starch concentrations, the free glucose concentration remains below 20 $g.l^{-1}$ during the whole cultivation period for all nitrogen sources; only the results with urea are shown in Figures 2a and b.

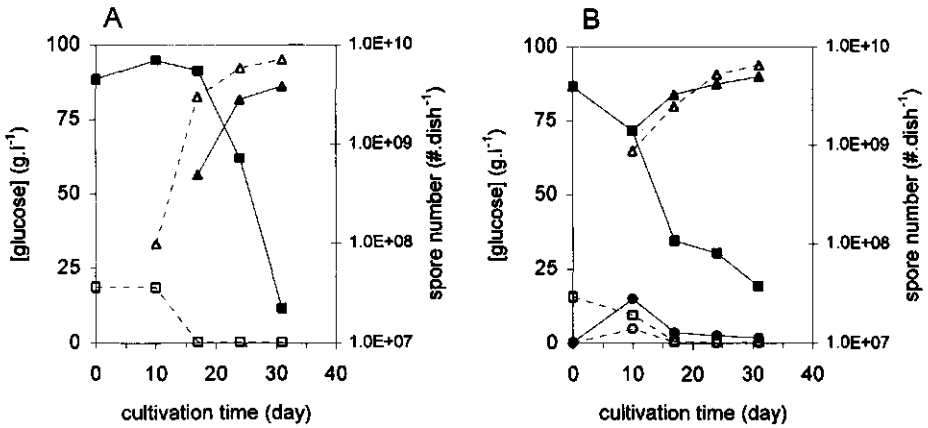

Figure 2: Time courses of spore production (▲), total glucose concentration (■) and free-glucose concentration (●) with urea combined with glucose (a) or starch (b) at low (dashed lines, open symbols) or high concentrations (solid lines, closed symbols).

From Figures 1 and 2 and the glucose measurements (data not shown) can be concluded that spore numbers are equal (urea and glycine combined with starch) or even lower at the higher substrate concentrations. Though more substrate is used at the higher substrate concentrations (usage 70 $g.l^{-1}$ vs. 20 $g.l^{-1}$ in case of urea, see Figure 2a and b). Visual evaluation of the Petri dishes has shown that in case of high substrate concentrations more mycelium is formed than with low substrate concentrations. High substrate concentrations can also lead to the formation of by-products just as with *Saccharomyces cerevisiae* where high glucose concentration or oxygen depletion leads to ethanol production. Further research is required to evaluate if high

substrate concentration leads to more mycelium formation or formation of by-products. Elemental balances might give an answer to this question, which is subject of Chapter 5.

CONCLUSION

Summarizing the experimental results, it can be stated that urea and glycine are the preferred nitrogen sources. With these nitrogen sources the pH during cultivation remains stable and high concentrations are not inhibiting when they are used in combination with starch. Since glycine can be used as carbon source as well by the fungus, urea is the preferred nitrogen source when elemental balances are aimed at. Starch is preferred to glucose, since substrate inhibition was shown to be less with starch. Therefore, future research will focus on evaluating the influence of other medium components on sporulation using starch as carbon source and urea as nitrogen source.

ACKNOWLEDGEMENTS

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REFERENCES

Engelkes, C.A., Nuclo, R.L. and Fravel, D.R. 1997. Effect of carbon, nitrogen, and C:N ratio of growth, sporulation, and biocontrol efficacy of *Talaromyces flavus*. Phytopathol. 87, 500-505.

Hallsworth, J.E. and Magan, N. 1994. Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. Microbiology, 140, 2705-2713.

Jackson, M.A. and Bothast, R.J. 1990. Carbon concentration and carbon-to-nitrogen ratio influence submerged-culture conidiation by the potential bioherbicide *Colletotrichum truncatum* NRRL 13737. Appl.Environ. Microb., 56, 3435-3438.

Jennings, D.H. 1995. The Physiology of Fungal Nutrition. 1st edition. University Press, Cambridge

Larroche, C. 1996. Microbial growth and sporulation behaviour in solid-state fermentation. J. Sci. Ind. Res., 5, 408-423.

McQuilken, M.P. and Whipps, J.M. 1995. Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol., 101, 101-110.

McQuilken, M.P., Budge, S.P. amd Whipps, J.M. 1997a. Effects of culture media and environmental factors on conidial germination, pycnidial production and hyphal extension of *Coniothyrium minitans*. Mycol. Res., 101, 11-17.

McQuilken, M.P., Budge, S.P. and Whipps, J.M. 1997b. Production, survival and evaluation of liquid culture-produced inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Biocontrol Sci. Technol., 7, 23-36.

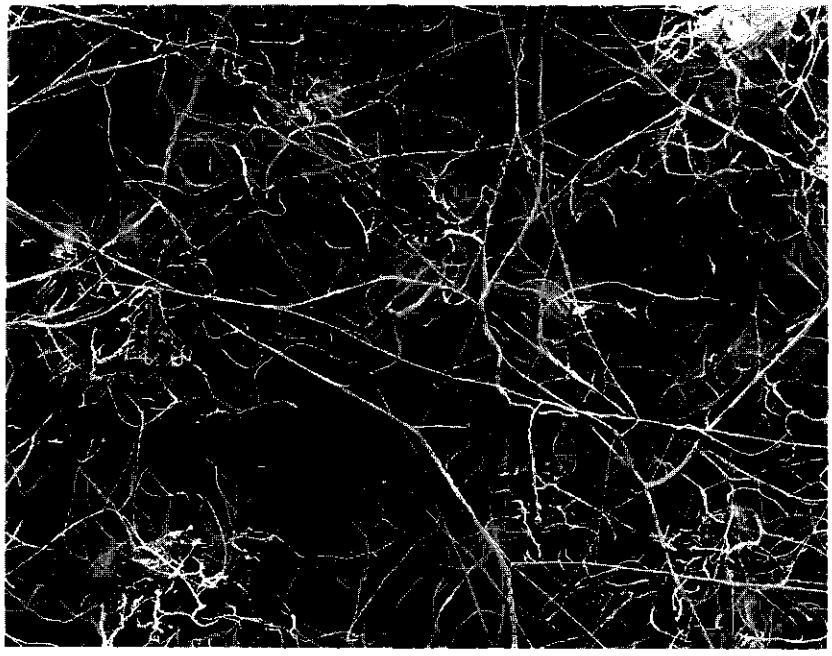
Ooijkaas, L.P., Tramper, J. and Buitelaar, R.M. 1998. Biomass estimation of *Coniothyrium minitans* in solid-state fermentation. Enzyme Microb. Technol. 22, 480-486.

Stryer, L. 1975. Biochemistry. W.H. Freeman and Company, New York.

Zhou, T., Reeleder, R.D. and Sparace, S.A. 1996. Influence of nutrients on growth of *Epicoccum nigrum*. Can. J. Microbiol., 42, 647-654.

CHAPTER 4

MEDIUM OPTIMIZATION FOR SPORE PRODUCTION OF *CONIOTHYRIUM MINITANS* USING STATISTICALLY BASED EXPERIMENTAL DESIGNS



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ABSTRACT

Statistically based experimental designs were used to optimize a chemically defined solid medium for the spore production of *Coniothyrium minitans*. In the first optimization step the influence of starch, urea, phosphate, magnesium, calcium, thiamin and trace elements on spore production was evaluated using a fractional factorial design. Starch and trace elements influenced spore production positively while urea affected spore production negatively. The other components had no significant influence on spore production. In the second and third steps the concentrations of starch, urea and trace elements were further optimized using central composite designs and response surface analysis. This optimization strategy allowed the spore production to be increased by a factor 7 from $4*10^9$ to almost $3*10^{10}$ spores per Petri dish of 9 cm diameter.

INTRODUCTION

Interest in "biopesticides" or "biocontrol agents" has recently increased as a result of the growing concern about the undesirable side effects of the chemical pesticides used at present. The fungus *Coniothyrium minitans* is an example of a promising biocontrol agent of *Sclerotinia sclerotiorum* which causes severe crop losses of a wide range of plants (Campbell, 1947; McQuilken and Whipps, 1995; Trutmann et al, 1980; Whipps and Gerlagh, 1992). As with other biopesticides, large numbers of infective spores of *C. minitans* are required for commercial application as biocontrol agent. Therefore, reliable mass production systems are needed.

Spore production of *C. minitans* on laboratory scale has been successfully achieved in solid-substrate fermentation (SSF) using several natural media, extracts and agar media (McQuilken and Whipps, 1995; McQuilken et al, 1997; Ooijkaas et al, 1998a). Although good results have been obtained, insufficient information is available on the influence of culture conditions on the quantity of spores produced for industrial purposes. Determining the optimal conditions for

conidia production of *C. minitans* is one key factor to develop a large-scale solid-substrate fermentation process.

However, information on the influence of nutritional and environmental conditions on mycelial growth and sporulation of *C. minitans* is sparse. Effects of temperature and water activity have been described by several researchers (Campbell, 1947; Turner and Tribe, 1976; McQuilken et al, 1997; Whipps and Magan, 1987; Magan and Whipps, 1988; Trutmann et al, 1980). More recently the effects of culture media on the mycelial growth, the number of pycnidia (bodies in which the spores arise) produced and the number of spores produced were investigated (McQuilken et al, 1997; Ooijkaas et al, 1998a). Nevertheless, the un- or semi-defined nature of the media used in these studies makes it difficult to determine which medium components affect spore production. To overcome this problem Ooijkaas et al (1998b) used a chemically defined solid medium to investigate the influence of several carbon and nitrogen sources. Effects of both source and concentration are seen (Ooijkaas et al, 1998b) but more detailed information on the influence of medium composition on sporulation of *C. minitans* is needed in order to optimize the spore production process.

Optimization of media is traditionally done by varying one factor while keeping the other factors at a constant level; the one-factor-at-a-time technique. This technique is tedious when a large number of factors have to be investigated, whereas statistically based experimental designs are a more efficient approach to deal with a large number of variables. Moreover if there are statistical interactions between factors, that is where the effect of one factor is dependent on the value of another factor, then this information will not be obtained using the one-factor-at-a-time technique.

Several researchers have used statistically based experimental designs to evaluate the influence of medium components in liquid fermentation on, e.g. enzyme production (Hounsa et al, 1996), production of certain metabolites (García-Ochoa et al, 1992; Zhang et al, 1996) biomass production (Lhomme and Roux, 1991) and spore production (Yu et al, 1997). Srinivas and co-workers (1994) and Zhu and co-workers (1996) used statistically based experimental designs to optimize enzyme production in solid-state fermentation.

The aim of this study was to optimize the spore production of *C. minitans* with respect to spore quantities on chemically defined solid medium with the aid of experimental designs. In the first optimization step a fractional factorial design was used to determine the influence on spore production of *C. minitans* of the following medium components: starch, urea, phosphate, calcium, magnesium, thiamin and trace elements. These components had earlier been found to influence spore production of other fungi (Larroche, 1996). In the following optimization steps the concentrations of the components which have a significant influence on sporulation were further optimized using central composite designs and response surface analysis.

MATERIALS AND METHODS

Microorganism, inoculum preparation

The microorganism used was *C. minitans* isolate IVT-1 (CBS 14896), kindly provided by Dr. M. Gerlagh from the IPO-DLO Research Institute for Plant Protection, Wageningen, The Netherlands. This isolate was chosen since this isolate, tested as reference G8, was grouped into colony type 1, which produces many pycnidia and spores (Sandy-Winsch et al, 1993) and this isolate, again tested as reference G8, gave promising results in bioassays (Gerlagh et al, 1996).

A spore stock suspension was obtained by growing *C. minitans* on potato dextrose agar (PDA, Difco) at 20 °C for 14 days. The conidia were harvested from the surface by adding sterile milliQ water and scraping the surface with a sterile spatula. The spore suspension obtained was counted using a Neubauer counting chamber, adjusted to $5*10^6$ spores.ml^{-1}, made up to 15% (w.v^{-1}) glycerol and stored as 1.5 ml aliquots at -80 °C.

Media, inoculation and incubation

The media tested contained the following components in different concentrations: starch, urea, KH_2PO_4, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, thiamin, trace elements solution, Mes (2-[n-morpholino]ethanesulfonic acid) and agar. The trace elements stock solution consisted of (in

g.l^{-1}): EDTA 1, $ZnSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.5, $Na_2MoO_4.2H_2O$ 0.02, $CuSO_4.5H_2O$ 0.02, $CoCl_2.6H_2O$ 0.04 and $MnCl_2.4H_2O$ 0.1. All the minerals present in the trace element stock solution are able to improve sporulation of fungi (Larroche et al, 1996). But for practical reasons, we tested the minerals as 'one component' and not as single components. Only the minerals phosphate, calcium and magnesium were separately tested. Thiamin was tested since preliminary experiments with *C. minitans* have shown that without this vitamin no sporulation occurs. Starch and urea were chosen as carbon and nitrogen source respectively based on previous results with *C. minitans* (Ooijkaas et al, 1998b). Mes was used since this buffer is one of the so-called 'Good-buffers' which hardly interfere with the cations present in the media (Good et al, 1966).

Stock solutions of these medium components were separately sterilized at 121 °C for 20 minutes except the thiamin solution that was filter sterilized (0.22 μm). Prior to sterilization the Mes stock solution was adjusted to pH 6.3 with NaOH. Due to sterilization a small part of urea (max. 3%) was transformed into volatile NH_3.

After being sterilized, the stock solutions were mixed to give final concentrations of 15 g.l^{-1} agar, 150 mM Mes and for the other components the final concentrations as required in the fractional factorial design (see Tables I and II) in the first optimization step. In the second and the third optimization steps the final concentrations were for agar 15 g.l^{-1}, Mes 150 mM, KH_2PO_4 2.0 g.l^{-1}, $CaCl_2.2H_2O$ 1 mg.l^{-1}, $MgCl_2.6H_2O$ 0.1 g.l^{-1}, thiamin 2 mg.l^{-1} and for starch, urea and trace elements as required in the central composite designs (Table III, IV and V). Sterile Petri dishes (9 cm diameter) were filled with 20 ml of the final media solutions.

Petri dishes with 20 ml agar media were inoculated with 100 μl of 10 times diluted spore stock suspension which were homogeneously spread over the agar surface with a sterile spatula. The dishes were incubated at 20 °C. To protect the plates against drying out, boxes filled with water were placed in the incubator to keep the air humid inside the incubator.

Sampling

At regular intervals after inoculation, two replicate Petri dishes of each media were removed from the incubator. All analyses were done after the whole content of each Petri dish was blended for 1 minute at full speed with 40 ml demineralized water using a laboratory Waring blender. The pH of each sample was measured and the number of spores in each blend was determined using a Neubauer counting chamber. Each experimental design was repeated. The data presented here are the means of the two duplicate experimental designs.

Experimental designs and data analysis

Fractional factorial design

The purpose of the first optimization step was to identify which component(s) of this defined medium have a significant effect on spore production of *C. minitans*. Factorial designs, one class of experimental designs, are very useful in identifying the important nutrients and interactions between two or more nutrients in relatively few experiments as compared to the one-factor-at-a-time technique (Cochran and Cox, 1957). Factorial designs require 2^N experiments if N factors have to be investigated. In our case, with seven variables, this would lead to 128 experiments, which is still a large number.

The number of experiments can be reduced by using only part of the factorial design (fractional factorial design) without loss of information about the main effects. However, some information about interaction effects will be lost (Box et al, 1978). In our case, a fractional factorial design was chosen since the higher-order interactions were expected to be negligible. We chose to do 1/8 of the 128 experiments, giving 16 experiments and for practical reasons to arrange the experiments in two blocks (Cochran and Cox, 1957). In each block of media a center point (CP) was added as an internal standard. The experimental design of this first optimization step is given in Table I with the levels in coded units. Table II gives the levels in natural units.

Table I: Fractional factorial design for 7 variables

Variable	Medium	A	B	C	D	E	F	G
Block 1:	1	-	-	-	-	-	-	-
	2	+	+	+	+	-	-	-
	3	+	+	-	-	+	+	-
	4	+	-	+	-	+	-	+
	5	+	-	-	+	-	+	+
	6	-	+	+	-	-	+	+
	7	-	+	-	+	+	-	+
	8	-	-	+	+	+	+	-
	CP[1]	0	0	0	0	0	0	0
Block 2:	9	+	+	-	-	-	-	+
	10	+	-	+	-	-	+	-
	11	+	-	-	+	+	-	-
	12	-	+	+	-	+	-	-
	13	-	+	-	+	-	+	-
	14	-	-	+	+	-	-	+
	15	-	-	-	-	+	+	+
	16	+	+	+	+	+	+	+
	CP[1]	0	0	0	0	0	0	0

[1]CP = center point

Table II: Levels of the variables tested in the fractional factorial design

			Coded level		
Variable	Component	Unit	+	-	0
A	Starch	$g.l^{-1}$	90	18	54
B	Urea	$g.l^{-1}$	10	2	6
C	KH_2PO_4	$g.l^{-1}$	4	1	2.5
D	$CaCl_2.2H_2O$	$mg.l^{-1}$	2	0.5	1
E	$MgCl_2.6H_2O$	$g.l^{-1}$	0.2	0.05	0.1
F	Thiamin	$mg.l^{-1}$	4	1	2
G	Trace elements	$ml.l^{-1}$	20	5	10

+ = high level, - = low level, 0 = center point level

The spore numbers were analyzed using Analysis of Variance (ANOVA) with the Genstat statistical package, version 5.32. To ensure that the assumptions of normality and constant variance were met, the response variable (spore numbers) was first transformed by taking the natural logarithm. The F-test was used to evaluate if a factor had a significant effect ($p \leq 0.05$).

Central composite design

Spore production of *C. minitans* can be written as a function (response surface) of the levels of the variables with a significant influence on spore production. The nature of this function is unknown but usually this kind of responses can be approximated by a second-order polynomial (Montgomery, 1976):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where Y = predicted response, β_0 = constant, β_i = coefficient for the linear effect, β_{ii} = coefficient for the quadratic effect, β_{ij} = coefficient for the interaction effect and x_i and x_j = the coded level of variable X_i and X_j.

The variables X_i were coded according to the following equation:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i}$$

where X_{cp} = the value of X_i at the center point level and ΔX_i = step change.

Central composite designs are useful designs to acquire data to fit this polynomial. To give the central composite design for three variables a 2^3-factorial design is combined with 6 replications of the center points and 6 axial points where one factor is set at an extreme level ($\pm \alpha$) and the other two factors are set at their center point level. For practical reasons the experiments were arranged in three blocks. To ensure that the blocks are orthogonal, which

simplifies the statistical analysis α was set at 1.633 (Cochran and Cox, 1957). This central composite design is given in Table III.

Table III: Central composite design for three variables in three blocks

		Coded level		
	Medium	starch	urea	trace elements
Block 1:	1	-1	-1	1
	2	1	-1	-1
	3	-1	1	-1
	4	1	1	1
	CP	0	0	0
	CP	0	0	0
Block 2:	5	-1	-1	-1
	6	1	-1	1
	7	-1	1	1
	8	1	1	-1
	CP	0	0	0
	CP	0	0	0
Block 3:	9	-1.633	0	0
	10	1.633	0	0
	11	0	-1.633	0
	12	0	1.633	0
	13	0	0	-1.633
	14	0	0	1.633
	CP	0	0	0
	CP	0	0	0

CP = center point

Both the coded and natural values of the levels tested of starch, urea and trace elements in the first and second central composite design are given in Tables IV and V, respectively.

After the natural logarithm of the spore numbers had been taken, these numbers were subjected to multiple regression analysis using the Genstat Program to obtain the coefficients of the second-order polynomial. The F-test was used to evaluate if the model was significant. By

analyzing the graphical representations of the equation, called response surfaces, the behavior of the model in the experimental area was investigated. The location of the optimum was determined by solving for all i the following set of equations:

$$-\beta_i = 2\beta_{ii}x_i + \sum \beta_{ij}x_j$$

Table IV: Levels of the variables tested in the first central composite design

		Coded level (x_i)					
Variable	unit	-1.633	-1	0	1	1.633	ΔX_i
Starch	$g.l^{-1}$	18.35	50	100	150	181.65	50
Urea	$g.l^{-1}$	0.53	1.00	1.75	2.50	2.97	0.75
Trace elements	$ml.l^{-1}$	3.7	10	20	30	36.3	10

Each level of variable X_i was coded according to:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i}$$

Table V: Levels of the variables tested in the second central composite design

		Coded level (x_i)					
Variable	unit	-1.633	-1	0	1	1.633	ΔX_i
Starch	$g.l^{-1}$	18.35	50	100	150	181.65	50
Urea	$g.l^{-1}$	2.96	3.75	5.00	6.25	7.04	1.25
Trace elements	$ml.l^{-1}$	27.3	40	60	80	92.6	20

Each level of variable X_i was coded according to:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i}$$

CHAPTER 4

RESULTS AND DISCUSSION

Fractional factorial design

In the first optimization step a 2^{7-3} fractional factorial design in two blocks was used (Cochran and Cox, 1957). In this way we could investigate the influence of seven medium components on spore production simultaneously in an experiment of practicable size. The effects on spore production of the following medium components were determined: starch, urea, phosphate, calcium, magnesium, thiamin and trace elements. The seven components mentioned were chosen based on preliminary experiments with *C. minitans* (see materials and methods) and results with other fungi (Larroche et al, 1996).

During cultivation of *C. minitans* on the 16 media and the center points of the fractional factorial design, the pH of the media slightly changes. In some media the pH drops from 6.3 to 6.0 and in other media the pH increases from 6.3 to 7.0. However, no correlation was found between medium composition, final pH and sporulation.

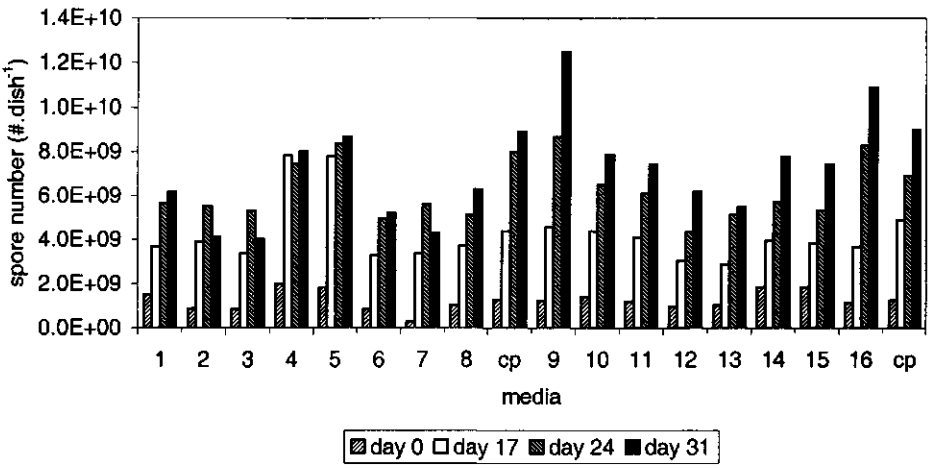

Figure 1: Spore production on the media of the fractional factorial design at different cultivation times

The results of the spore counts are given in Figure 1. As can be seen, medium composition affects spore production markedly. Media 9, 16 and the center points give the best results after 31 days of cultivation ($1.0\text{-}1.2*10^{10}$ spores.dish^{-1}) while media 2, 3 and 7 produce the lowest spore numbers ($4\text{-}5*10^{9}$ spores.dish^{-1}). Media 4 and 5 gave the best results after 17 days but no more spores are produced up to day 31 on these two media. Possibly, no more spores are produced since a substrate became exhausted, most likely urea, or a metabolite that inhibits sporulation was produced. However, no measurements were done so this remains speculative. The decline in spore numbers after 24 days on media 2, 3 and 7 is unexpected and the reason for this decline is unclear.

Statistical analysis of the data (F-test) showed that only starch, urea and trace elements in the concentration range tested had a significant effect on spore production ($p \leq 0.05$). When comparing the treatments means, the mean spore number at the low (-) level with the mean at the high (+) level, we see that spore production is negatively affected by urea ($7.26*10^{9}$ vs. $5.88*10^{9}$) and positively affected by starch ($5.84*10^{9}$ vs. $7.32*10^{9}$) or trace elements ($5.66*10^{9}$ vs. $7.52*10^{9}$).

The negative effect of urea on sporulation coincides with the fact that for some fungi nitrogen limitation induces sporulation, for example, *Aspergillus niger* and *Penicillium griseofulvum* sporulate after nitrogen depletion (Smith and Galbraith, 1971). Possibly sporulation of *C. minitans* takes place after nitrogen depletion too. If this is the case, low urea concentrations are more favorable than high urea concentrations.

The positive effect of starch might be caused by the availability of a higher amount of substrate to synthesize the spores. Sporulation is associated with the synthesis of macromolecules such as proteins, carbohydrates and RNA, which are needed to produce the sporulation-specific structures and storage molecules needed for germination (Dahlberg and van Etten, 1982). Therefore, high starch concentrations could be advantageous since a higher amount of substrate is available to synthesize these macromolecules and consequently more spores can be produced. However, the positive effect of starch is in contradiction with results of a previous study where the same starch concentrations were tested and high starch concentrations did not result in higher spore numbers (Ooijkaas et al, 1998b). Perhaps this difference in results is

caused since in the previous study a high urea concentration was used which influenced sporulation negatively (Ooijkaas et al, 1998b).

The addition of higher amounts of trace elements leads to higher spore numbers. A feasible explanation is that enzymes involved in the sporulation process may need trace elements as cofactor. Though, which trace element(s) is/are responsible for the increase in spore production of *C. minitans* is unknown since all trace elements present in the stock solution are able to improve fungal sporulation (Larroche, 1996).

Although no information was obtained on how medium components affect the mechanisms behind spore production, this fractional factorial design proved to be a valuable tool for screening which medium components in the concentration range tested had a significant influence on spore production of *C. minitans*. Nevertheless, it cannot predict the optimal levels of the medium components that significantly affect spore production. This information was obtained from the second optimization step.

Central composite design

In this second optimization step the levels of the three significant variables starch, urea and trace elements were further optimized using a central composite design (Cochran and Cox, 1957). Based on the results of the fractional factorial design where starch and trace elements had a positive effect and urea had a negative effect on spore production, the ranges of levels for starch, urea and trace elements were chosen. With this central composite design we tested higher levels of starch and trace elements and lower levels of urea (Table IV). The levels of the other nutrients were set at the level of the center point in the fractional factorial design since this center point medium gave high spore numbers after 31 days.

As can be seen from Figure 2, media 4 and 7 of this central composite design produced the highest number of spores while media 11 and 13 of this design produced the lowest number of spores. Spore numbers after 31 days of cultivation range from $4*10^9$ spores.dish^{-1} to $1.2*10^{10}$ spores.dish^{-1} which is in the same range of numbers as obtained in the fractional factorial design.

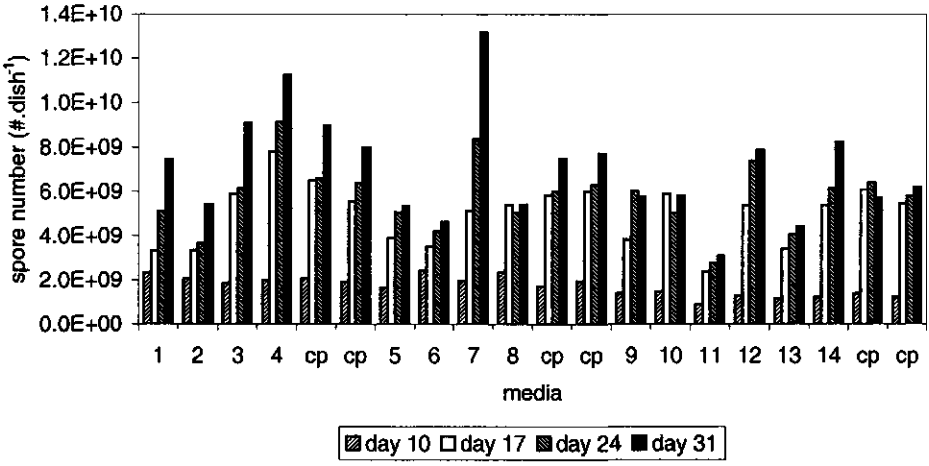

Figure 2: Spore production on the media of the first central composite design at different cultivation times

Regression analysis of the log-transformed data obtained after 31 days of cultivation resulted in the following second-order polynomial which describes the natural logarithm of the spore numbers produced per Petri dish (Y) as function of the coded levels of starch (x_1), urea (x_2) and trace elements (x_3):

$$Y = 22.6782 - 0.0825x_1 + 0.2596x_2 + 0.1768x_3 - 0.0148x^2_1 - 0.0748x^2_2 - 0.0089x^2_3 - 0.237x_1x_2 - 0.0188x_1x_3 + 0.1217x_2x_3$$

Statistical testing of this model reveals that this second-order model is significant at the 99% level and the model explains 84.8% (R_{adj}^2) of the total variation. This means that the model is an adequate predictor of the experimental results. The standard error of the observations (transformed spore numbers) was estimated to be 0.142.

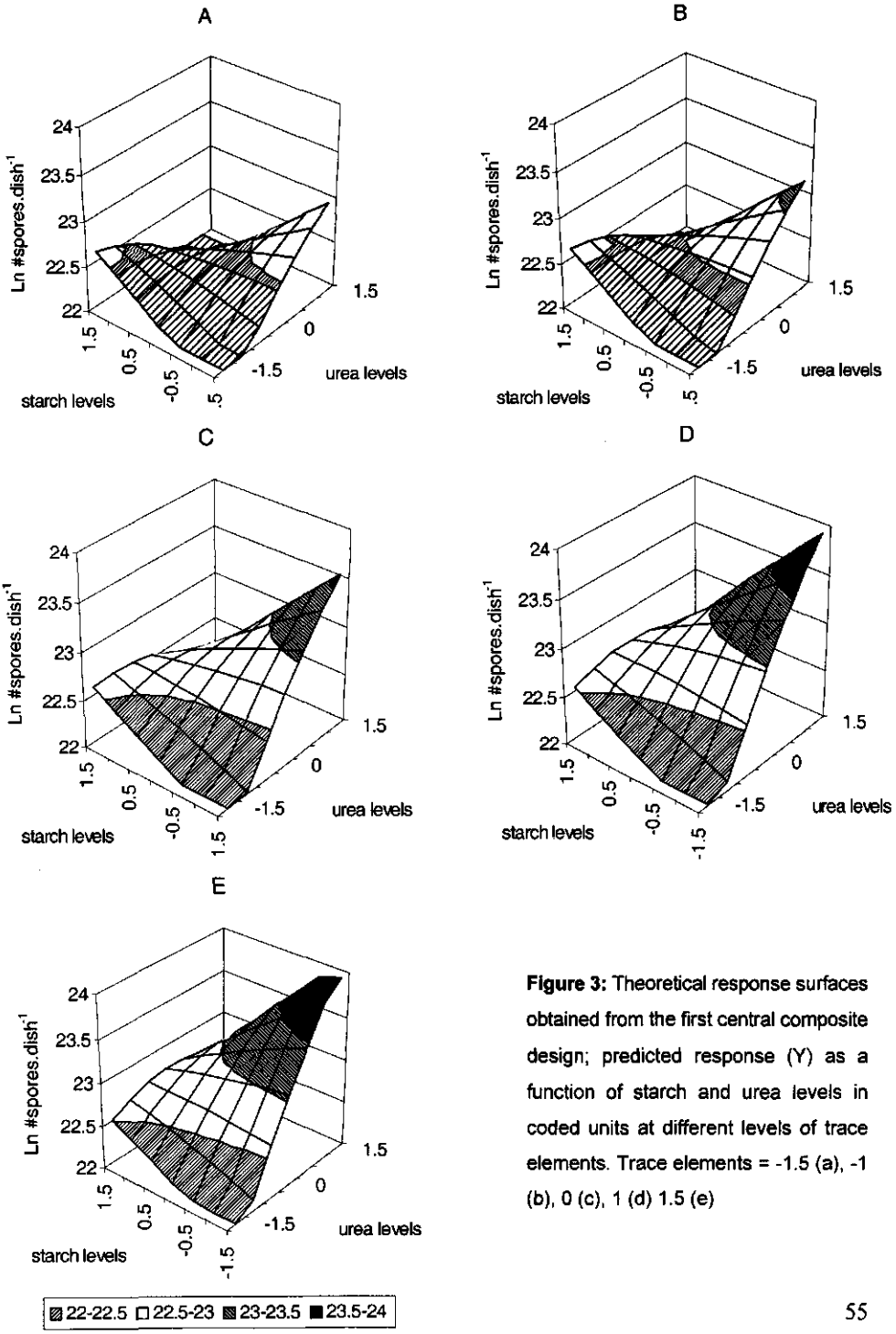

Figure 3: Theoretical response surfaces obtained from the first central composite design; predicted response (Y) as a function of starch and urea levels in coded units at different levels of trace elements. Trace elements = -1.5 (a), -1 (b), 0 (c), 1 (d) 1.5 (e)

From the analysis of the response surfaces (shown in Figures 3) it can be seen that there is no clear optimum within the experimental range investigated. This was confirmed by the solutions of the set of derivatives. The best spore production in this central composite design is obtained at the lowest level of starch and the highest levels of urea and trace elements as can be concluded from the response surface analyses shown in Figures 3.

This conclusion did not conform to the expectation we had from the fractional factorial design, namely, that using high levels of starch and trace elements and low levels of urea could enhance spore production. It appears that in this central composite design the levels tested for urea and trace elements were too low. For this reason and the fact that we did not find an optimum, a second central composite design was executed to determine the optimal levels for starch, urea and trace elements.

The levels of starch, urea and trace elements in this second central composite design (Table V) were chosen based on the information obtained in the fractional factorial and first central composite design. Although in the first central composite design the lowest level of starch was the best for spore production (Figures 3), the levels for starch in this second central composite design remain the same as in the first central composite design. The levels were kept the same since in the fractional factorial design spore production was just enhanced by raising the starch concentration. Spore production was the best at the lowest level of the fractional factorial design and at the highest level of urea in the first central composite design (Figures 3). The latter level is higher than the lowest level in the fractional factorial design. Therefore, urea levels were chosen between the highest level of the first central composite design and the highest level of the fractional factorial design. In both the fractional factorial and the first central composite design, spore production was enhanced by using high amounts of trace elements (Figures 3) hence, even higher levels for trace elements were tested in this second central composite design.

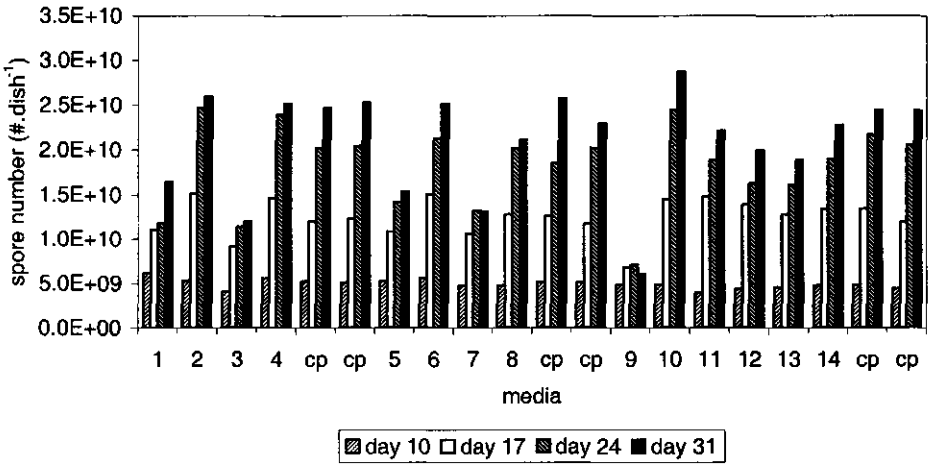

Figure 4: Spore production on the media of the second central composite design at different cultivation times

The results of this second central composite design are given in Figure 4. Spore numbers after 31 days of cultivation range from $6*10^9$ spores.dish^{-1} to $2.9*10^{10}$ spores.dish^{-1} which is a considerable improvement as compared to the results of the fractional factorial and the first central composite design (max. $1.2*10^{10}$ spores.dish^{-1}). Using the transformed data acquired after 31 days of cultivation the following function was obtained:

$$Y = 23.9197 + 0.3530x_1 - 0.0632x_2 + 0.0460x_3 - 0.2165x^2_1 - 0.0419x^2_2 - 0.0477x^2_3 + 0.0339x_1x_2 + 0.0013x_1x_3 + 0.0287x_2x_3$$

Being significant at the 99% level and explaining 89.6% of the variance (R_{adj}^2), this model gives a good approximation of the true response. The standard error of the observations was 0.118.

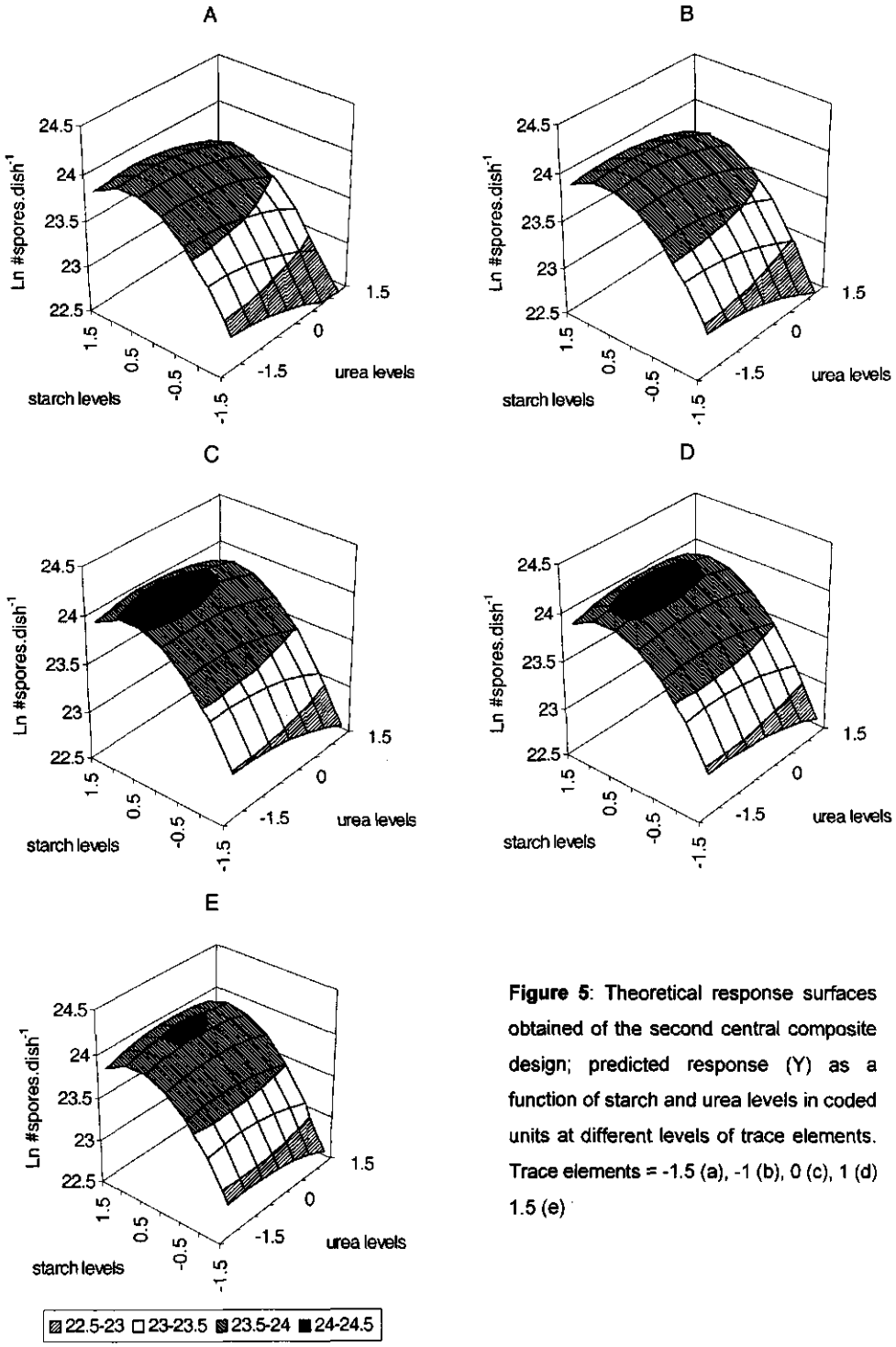

Figure 5: Theoretical response surfaces obtained of the second central composite design; predicted response (Y) as a function of starch and urea levels in coded units at different levels of trace elements. Trace elements = -1.5 (a), -1 (b), 0 (c), 1 (d) 1.5 (e)

Response surface analysis (Figures 5) indicates that a local optimum exists in the area experimentally investigated. The optimal levels in coded values for starch lie between 0 and 1, for urea between -1 and 0 and for trace elements between 0 and 1. The solutions of the set of derivatives reveal that the optimum values in coded units for spore production of *C. minitans* are 0.793 (139.67 $g.l^{-1}$) for starch, -0.295 (4.63 $g.l^{-1}$) for urea and for trace elements 0.404 (68.09 $ml.l^{-1}$) with the corresponding Y = 24.078 ($2.86*10^{10}$ $spores.dish^{-1}$). Practical validation of this theoretical optimum medium gave spore numbers of $2.4*10^{10}$ spores per Petri dish. Although in practice this optimum medium produces a lower amount of spores than predicted, this spore number is still a very good result. In the past, with this kind of production system spore numbers of about $5*10^{9}$ spores per Petri dish of 9 cm diameter are obtained (Ooijkaas et al, 1998 a and b).

Central composite designs and response surface analysis were useful tools to determine the optimum levels of starch, urea and trace elements for spore production of *C. minitans*. Although the effects of starch, urea and trace elements on sporulation were evaluated, the underlying mechanisms by which these components affect sporulation of *C. minitans* are still unknown. Further research is required to elucidate these mechanisms.

CONCLUSIONS

Statistically based experimental designs proved to be valuable tools in optimizing a chemically defined solid medium for spore production of *C. minitans*. Fractional factorial design used in the first step was an efficient approach to screen which medium components affect spore production significantly. Central composite designs and response surface analysis used in the second and third steps were useful to determine the optimum levels of the components that significantly influence sporulation.

The final composition of the chemically defined solid media to produce spores of *C. minitans* after the three optimization steps is: agar 15 $g.l^{-1}$, Mes 150 mM, starch 139.67 $g.l^{-1}$, urea 4.63 $g.l^{-1}$, KH_2PO_4 2.0 $g.l^{-1}$, $CaCl_2.2H_2O$ 1 $mg.l^{-1}$, $MgCl_2.6H_2O$ 0.1 $g.l^{-1}$, thiamin 2 $mg.l^{-1}$ and trace

elements stock solution 68.09 ml.l^{-1}. This media produces theoretically 2.86*10^{10} spores.dish^{-1} and in practice 2.42*10^{10} spores.dish^{-1} which is a considerable improvement as compared to the results of previous studies.

ACKNOWLEDGMENTS

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REFERENCES

Box, G.E.P., Hunter W.G. and Hunter, J.S. 1978. Statistics for experimenters. John Wiley and Sons, New York.

Campbell, W.A. 1947. A new species of *Coniothyrium* parasitic on sclerotia. Mycologia 39, 190-195.

Cochran, W.G. and Cox, G.M. 1957. Experimental designs, 2nd edition. John Wiley and Sons, New York.

Dahlberg, K.R. and van Etten, J.L. 1982. Physiology and biochemistry of fungal sporulation. Ann. Rev. Phytopathol. 20, 281-301.

García-Ochoa, F., Santos, V.E. and Fritsch, A.P. 1992. Nutritional study of *Xanthomonas campestris* in xanthan gum production by factorial design of experiments. Enzyme Microb. Technol. 14, 991-996.

Gerlagh, M., Whipps, J.M., Budge, S.P. and Goossen-van de Geijn, H.M. 1996. Efficiency of isolates of *Coniothyrium minitans* as mycoparasites of *Sclerotinia sclerotiorum*, *Sclerotinia cepivorum* and *Botrytis cinerea* on tomato stem pieces. Eur. J. Plant Pathol. 102, 787-793.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S. and Singh, R.M.M. 1966. Hydrogen ion buffers for biological research. Biochemistry, 5, 467-477.

Hounsa, C.G., Aubry, J.M., Dubourguier, H.C. and Hornez, J.P. 1996. Application of factorial and Doehlert designs for optimization of pectate lyase production by a recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. 45, 764-770.

Larroche, C. 1996. Microbial growth and sporulation behaviour in solid-state fermentation. J. Sci. Ind. Res. 55, 408-423.

Lhomme, B. and Roux, J.C. 1991. Utilization of experimental designs for optimization of *Rhizopus arrhizus* culture. Biores. Technol. 35, 301-312.

Magan, N. and Whipps, J.M. 1988. Growth of *Coniothyrium minitans*, *Gliocladium roseum*, *Trichoderma harzianum* and *T. viride* from alginate pellets and interaction with water availability. EPPO Bulletin 18, 37-45.

McQuilken, M.P., Budge, S.P. and Whipps, J.M. 1997. Effects of culture media and environmental factors on conidial germination, pycnidial production and hyphal extension of *Coniothyrium minitans*. Mycol. Res. 101, 11-17.

McQuilken, M.P. and Whipps, J.M. 1995. Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol. 101, 101-110.

Montgomery, D.C. 1976. Design and analysis of experiments, 2nd edition. John Wiley and Sons, New York.

Ooijkaas, L.P., Chin-Joe, I, Tramper, J. and Buitelaar, R.M. 1998b. Spore production of *Coniothyrium minitans* during solid-state fermentation on different nitrogen sources with glucose of starch as carbon source. Biotechnol. Lett 20, 785-788

Ooijkaas, L.P., Tramper, J. and Buitelaar, R.M. 1998a. Biomass estimation of *Coniothyrium minitans* in solid-state fermentation. Enzyme Microb. Technol. 22, 480-486.

Sandys-Winsch, C., Whipps, J.M., Gerlagh, M. and Kruse, M. 1993. World distribution of the sclerotial mycoparasite *Coniothyrium minitans*. Mycol. Res. 97, 1175-1178.

Smith, J.E. and Galbraith, J.C. 1971. Biochemical and physiological aspects of differentiation in the fungi. Adv. Microbiol. Physiol. 5, 45-134.

Srinivas, M.R.S., Chand, N. and Lonsane B.K. 1994. Use of Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promotors, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid-state fermentation. Bioprocess Eng. 10, 139-144.

Trutmann, P., Keane, P.J. and Merriman, P.R. 1980. Reduction of sclerotial inoculum of *Sclerotinia sclerotiorum* with *Coniothyrium minitans*. Soil Biol. Biochem. 12, 461-465.

Turner, G.J. and Tribe, H.T. 1976. On *Coniothyrium minitans* and its parasitism of *Sclerotinia* species. Trans. Br. Mycol. Soc. 66, 97-105.

Whipps, J.M. and Gerlagh, M. 1992. Biology of *Coniothyrium minitans* and its potential for use in disease biocontrol. Mycol. Res. 96, 897-907.

Whipps, J.M. and Magan, N. 1987. Effects of nutrient status and water potential of media on fungal growth and antagonist-pathogen interactions. EPPO Bulletin 17, 581-591.

Yu, X., Hallet, S.G., Sheppard, J. and Watson, A.K. 1997. Application of the Plackett-Burman experimental design to evaluate nutritional requirements for the production of *Colletotrichum coccodes* spores. Appl. Microbiol. Biotechnol. 47, 301-305.

Zhang, J., Marcin, C., Shifflet, M.A., Salmon, P., Brix, T., Greasham, R., Buckland, B. and Chartrain, M. 1996. Development of a defined medium fermentation process for physotigmine production by *Streptomyces griseofuscus*. Appl. Microbiol. Biotechnol. 44, 568-575.

Zhu, Y., Knol, W., Smits, J.P.and Bol, J. 1996. Medium optimization for nuclease P1 production by *Penicillium citrinum* in solid-state fermentation using polyurethane foam as inert carrier. Enzyme Microb. Technol. 18, 108-112.

CHAPTER 5

GROWTH AND SPORULATION STOICHIOMETRY AND KINETICS OF *CONIOTHYRIUM MINITANS* IN SOLID-STATE FERMENTATION

This chapter is submitted for publication.

CHAPTER 5

ABSTRACT

Coniothyrium minitans was cultivated on agar media with different concentrations of starch, urea and trace elements. By means of elemental balances the stoichiometry of growth and sporulation was established. It was concluded that *C. minitans* produces by-products on all media, especially in the medium with high urea concentrations where 30% of the starch is converted into by-products.

Simple empirical models were used to describe the kinetics of growth, sporulation, carbon dioxide production and substrate consumption on all media. Total biomass and mycelium could be described reasonably well with the logistic law. Starch, urea and oxygen consumption, and carbon dioxide production could be described as function of total biomass by the linear-growth model of Pirt. There were almost no differences between media for the estimates of the yield coefficients and maintenance coefficients. Only at high urea concentration maintenance coefficients were much higher. Similar to substrate consumption and carbon dioxide production, the kinetics of sporulation could be described as function of mycelium production with the linear-growth model. It was shown that sporulation of *C. minitans* was growth-associated.

Based on the kinetics, the process costs for producing spores were roughly calculated. It was shown that fermentor costs are the major part of the production costs.

INTRODUCTION

During recent years, the use of fungal spores for the biological control of plant pests and diseases has received increasing interest. One promising fungus is *Coniothyrium minitans*, a natural antagonist of the plant pathogen *Sclerotinia sclerotiorum* (Campbell, 1947; McQuilken and Whipps, 1995; Whipps and Gerlagh, 1992; Gerlagh et al, 1999). Mass production of spores is an essential step to commercialize this biological control agent; hence, reliable mass production methods are needed. Since small spore numbers are

obtained in liquid cultures while high numbers are produced by solid-state cultivation, a solid-state fermentation (SSF) process is the preferred mass production method for this fungus (McQuilken et al, 1997a; Ooijkaas et al, 1998a,b; Ooijkaas et al, 1999; Weber et al, 1999).

For the optimal design and control of a solid-state fermentation process, mathematical models are useful tools. These models require quantitative estimates of growth and sporulation of *C. minitans* on solid substrates and information on the stoichiometry of the bioconversion reactions. Knowledge of the stoichiometry and kinetic parameters are essential to be able to predict the rates of biomass production, spore production, substrate and oxygen consumption. The latter is a direct indicator for metabolic heat production, which is of great importance for control of SSF. Moreover, knowledge of growth and sporulation kinetics can extend our understanding of the biological behavior of this fungus.

Until now, hardly any information concerning the stoichiometry and kinetics of growth and sporulation of *C. minitans* is available, despite the studies on the effect of environmental conditions on growth and sporulation (McQuilken et al, 1997b; Ooijkaas et al, 1998b; Ooijkaas et al, 1999). Therefore the present study was undertaken.

The stoichiometry of mycelium and spore production can be derived from the composition of substrates and biomass and elemental balances. This approach has been used successfully to identify nutrient limitations of *Penicillium roqueforti* growing on buckwheat (Larroche and Gros, 1992). A similar approach was published for characterization of growth and sporulation of *Metarhizium anisopliae* (Dorta et al, 1996). In addition, elemental balances can be used to determine if other by-products besides mycelium and spores are formed.

Previous research with *C. minitans* suggested that high carbon and nitrogen concentrations lead to more mycelium and/or by-products instead of more spores (Ooijkaas et al, 1998b). To evaluate if excess of substrate leads to more mycelium or by-products, the C- and N-balances are set up in the present study. Furthermore, estimates of kinetic parameters like specific growth rate, yield and maintenance coefficients are obtained from simple mathematical expressions describing the experimentally determined growth and spore

production. In addition, preliminary cost calculations will be made based on the estimates of kinetic models.

MATERIALS AND METHODS

Microorganism, inoculum preparation

The microorganism used was *C. minitans* isolate IVT-1 (CBS 14896), kindly provided by Dr. M. Gerlagh from the IPO-DLO Research Institute for Plant Protection, Wageningen, The Netherlands. A spore stock suspension was obtained as follows. *C. minitans* was grown on potato dextrose agar (PDA, Difco) at 20 °C for 14 days. The conidia were then harvested from the surface by adding sterile milliQ water and scraping the surface with a sterile spatula. The spore suspension obtained was counted using a Neubauer counting chamber, adjusted to $5*10^6$ spores.ml^{-1}, made up to 15% (w.v^{-1}) glycerol and stored as 1.5 ml aliquots at -80 °C.

Media, inoculation and incubation

The media tested contained the following components: starch, urea, KH_2PO_4, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, thiamin, trace elements solution, Mes (2-[n-morpholino]ethanesulfonic acid) and agar. The trace elements stock solution consisted of (in g.l^{-1}): EDTA 1, $ZnSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.5, $Na_2MoO_4.2H_2O$ 0.02, $CuSO_4.5H_2O$ 0.02, $CoCl_2.6H_2O$ 0.04 and $MnCl_2.4H_2O$ 0.1. Stock solutions of these medium components were separately sterilized at 121 °C for 20 minutes except the thiamin solution that was filter sterilized (0.22 µm). Prior to sterilization the Mes stock solution was adjusted to pH 6.3 with NaOH. Control measurements reveal that due to sterilization a small part of urea (max. 3%) was transformed into volatile NH_3; however, this had no effect on the pH.

After being sterilized, the stock solutions were mixed to give final concentrations of agar 15 g.l^{-1}, Mes 150 mM, KH_2PO_4 2.0 g.l^{-1}, $CaCl_2.2H_2O$ 1 mg.l^{-1}, $MgCl_2.6H_2O$ 0.1 g.l^{-1}, thiamin 2

mg.l^{-1} and for starch, urea and trace elements as given in Table I. Sterile Petri dishes (9 cm diameter) were filled with 20 ml of the final media solutions.

Table I: Concentrations of starch urea and trace elements for the different media

Media	starch (g.l^{-1})	urea (g.l^{-1})	trace elements (ml.l^{-1})
D1[1]	18	2	10
D2[1]	90	10	10
D3[2]	140	4	70

[1] concentrations as used before in screening experiments; starch combined with urea at low (D1) and high concentrations (D2) (Ooijkaas et al, 1998b)

[2] concentrations after medium optimization with statistically based experimental designs (Ooijkaas et al, 1999)

Petri dishes with 20 ml agar media were covered with a sterilized nylon membrane filter (Schleicher and Schuell, NY-13-N, 0.2 μm) and inoculated with 100 μl of 10 times diluted spore stock suspension; the spores were homogeneously spread over the filter surface with a sterile spatula. One Petri dish was placed in a desiccator (for gas analysis) with a volume of approximately 2.5 l filled with 0.1 l water to protect the plate against drying out (one desiccator per medium). The remaining dishes and the desiccators were incubated at 20 °C. To protect the plates against drying out boxes filled with water were placed in the incubator to keep the air humid inside the incubator.

Sampling

At regular intervals after inoculation, the amount of oxygen and carbon dioxide in the desiccator was determined by GC analysis before and after opening and closing the desiccator to replace the Petri dish by one from the incubator. The Petri dish from the desiccator was used for harvesting the spores and mycelium and further analysis.

Each experiment was repeated. The data presented here are the means of the two duplicate experiments.

Analysis

O_2 and CO_2

Gas analysis was done by injecting the gas into a micro gas chromatograph (CP-2002, Chrompack) fitted with an automatic sampling port and a thermal conductivity detector. Helium was used as carrier gas. A HayeSep A column (Chrompack) was used for CO_2 determination and a Molsieve 5Å PLOT column (Chrompack) for O_2 determination.

Harvest and dry weight

Harvesting of spores and mycelium was done by scraping the biomass from the filter with a spatula. The biomass was placed in a tube and the filter was washed twice with 10 ml demineralized water. This water was collected in the same tube as the biomass. Spores were extracted from the mycelium by vigorously shaking the tube. The suspension then obtained was filtered through two layers of cheesecloth to separate the spores (pass the cloth) from the mycelium (stays behind). The extraction procedure was repeated several times with the mycelial residue and 10 ml demineralized water until the filtrate was clear. The mycelial residue was then transferred into pre-weighed Eppendorf tubes and stored at –80 °C before freeze-drying.

The filtrates were collected in a pre-weighed centrifuge tube and centrifuged for 5 min at 1500 g. The spores were re-suspended in water and centrifuged as above. The tube with pellet was then stored at –80 °C before freeze-drying. After freeze-drying, the pre-weighed (Eppendorf) tubes were re-weighed for dry weight determination of the lyophilized material.

Elemental composition

The elemental composition (C, H and N) of mycelium and spores was determined by GC (Carlo Erba Instruments). The percentage O in the biomass was estimated by difference (100-(C+H+N+ash content)). The ash content was assumed to be 5% for mycelium and 3%

for spores based on results with *P. roqueforti* and *M. anisopliae* (Desfarges et al, 1987; Dorta et al, 1996).

Starch and urea

Starch and urea analyses were done after the agar medium of each Petri dish was blended for 1 minute at full speed with 40 ml demineralized water using a laboratory Waring blender. Starch was determined as the total amount of glucose after an enzymatic hydrolysis with amyloglucosidase. For that a 100 µl sample was incubated with 900 µl citric acid buffer (50 mM, pH 4.8) and 0.3 mg amyloglucosidase (75 $U.mg^{-1}$, Merck) for 2 h at 60 °C. After this hydrolysis step, the amount of glucose was measured with the enzymatic glucose-oxidase peroxidase assay (GOD-PAP, Merck) according to the manufacturer's protocol. Urea was measured with a commercially available colorimetric assay (640A, Sigma diagnostics) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Stoichiometry

The stoichiometry of mycelium and spore production of *C. minitans* can be derived from the composition of substrates and biomass and elemental balances. These elemental balances can be deduced from the reaction equation (eq. 1) for the conversion of starch ($CH_{1.667}O_{0.833}$), urea (CH_4N_2O) and oxygen to mycelium ($CH_{Am}O_{Bm}N_{Cm}$), spores ($CH_{Asp}O_{Bsp}N_{Csp}$), carbon dioxide, water and possibly by-products ($CH_{Ap}O_{Bp}N_{Cp}$).

$$CH_{1.667}O_{0.833} + y_n CH_4N_2O + y_o O_2 \rightarrow y_m CH_{Am}O_{Bm}N_{Cm} + y_{sp} CH_{Asp}O_{Bsp}N_{Csp} + y_c CO_2 + y_p CH_{Ap}O_{Bp}N_{Cp} + y_w H_2O \quad (1)$$

In this eq. 1, y_i stands for the stoichiometric coefficient or macroscopic yield factor for compound i on one C-mole starch ((C-)mole.C-$mole^{-1}$), A_i, B_i and C_i represent respectively

the hydrogen, oxygen and nitrogen content of one C-mole of compound i, where i is: urea (n), oxygen (o), mycelium (m), spores (sp), carbon dioxide (c), by-products (p) or water (w).

The stoichiometric coefficient for carbon dioxide y_c can be written as:

$$y_c = y_n + y_{cresp} \tag{2}$$

where y_n stands for the amount of carbon dioxide released by hydrolysis of urea and y_{cresp} for the amount of carbon dioxide due to fungal respiration.

The elemental analysis of mycelium and spores and the corresponding elemental formulas are given in Table II. As can be seen, these compositions vary with medium composition. Furthermore, the composition is not the same for mycelium and spores. Compared to the general elemental formula for biomass $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1983), the nitrogen content of this fungus is very low. However, the nitrogen content is similar to that for *P. roquefortii* (Larroche and Gros, 1992) and *M. anisopliae* (Dorta et al, 1996).

Table II: Elemental composition of biomass after 35 days of cultivation

		Elemental composition (% w.w^{-1})				
		C	H	O	N	Elemental formula
D1	mycelium	44.53	6.55	38.53	5.39	$CH_{1.75}O_{0.65}N_{0.10}$
	spores	51.36	6.99	33.00	5.65	$CH_{1.62}O_{0.48}N_{0.09}$
D2	mycelium	47.69	6.66	35.12	5.53	$CH_{1.66}O_{0.55}N_{0.10}$
	spores	54.37	7.52	29.84	5.28	$CH_{1.65}O_{0.41}N_{0.08}$
D3	mycelium	48.95	6.53	34.72	4.79	$CH_{1.59}O_{0.53}N_{0.08}$
	spores	55.23	7.33	30.15	4.29	$CH_{1.58}O_{0.41}N_{0.07}$

The stoichiometric coefficients y_n, y_o, y_m, y_{sp} and y_c are estimated from direct measurements as the total amount of compound i consumed or produced divided by the total amount of starch consumed at day 35. These values are given in Table III. If we include the values for the stoichiometric coefficients and the elemental composition of spores and mycelium in

the C and N-balances deduced from eq. 1 it is possible to calculate the average stoichiometric coefficient for unknown by-products (y_p) and the nitrogen content of these by-products (C_p). Based on these C and N-balances we conclude that in all media unknown by-products are formed.

Table III: Average stoichiometric coefficients or macroscopic yield factors (y_i) ((C-) mole C-mole^{-1}), nitrogen content (C_p) and degree of reduction for by-products (γ_p) over 35 days of cultivation

		D1	D2	D3
y_m	C-mol.C-mol^{-1}	0.04	0.09	0.10
y_{sp}	C-mol.C-mol^{-1}	0.32	0.18	0.33
y_o	mol.C-mol^{-1}	0.51	0.41	0.50
y_{cresp}	mol.C-mol^{-1}	0.48	0.41	0.51
y_n	mol.C-mol^{-1}	0.05	0.05	0.04
y_p[1]	C-mol.C-mol^{-1}	0.16	0.32	0.07
C_p[1]	N-mol.C-mol^{-1}	0.09	0.10	0.11
γ_p[2]		2.46	3.60	1.45
y_{enz}	C-mol.C-mol^{-1}	0.06	0.13	0.03
y_{oth}	C-mol.C-mol^{-1}	0.10	0.20	0.04
γ_{oth}		1.40	3.07	-0.74

[1] estimated from C- and N-balance

[2] estimated from the degree of reduction balance.

The values of the stoichiometric coefficients for unknown by products are 0.16, 0.32 and 0.07 (see Table III) corresponding with by-product concentrations of 0.11, 1.07 and 0.36 C-mol.l^{-1} for D1, D2 and D3 respectively. The high concentrations of by-products found on D2 explains why in previous research it was seen that on D2 more starch is used but no more spores are produced as compared to D1 (Ooijkaas et al, 1998b). The starch is converted into by-products rather than into spores on D2.

An indication on the nature of these by-products can be obtained from the degree of reduction, which is a measure of the number of electrons transferred to oxygen in the oxidation of one C-mole of compound i to CO_2, H_2O and N-source. The degree of reduction

for these by products (γ_p) can be calculated from the degree of reduction balance for growth and sporulation of *C. minitans* given by (Erickson et al, 1978):

$$\gamma_s - 4\gamma_o = y_x\gamma_x + y_{sp}y_{sp} + y_p\gamma_p \quad (3)$$

where γ_i is the degree of reduction for component i. For urea as nitrogen source γ_i is given by (Roels, 1983):

$$\gamma_i = 4 + A_i - 2B_i - 3C_i \quad (4)$$

The degree of reduction of the unknown by-products (γ_p) is given in Table III together with the values of the stoichiometric coefficient (y_p) and the nitrogen content (C_p) of these by-products.

As can be seen from Table III where all the stoichiometric coefficients are given, unknown by-products are formed in all media, relatively high amounts in D2 and relatively low amounts in D3. Part of these by-products consists of extracellular enzymes, e.g. amylases and urease, otherwise starch and urea could not be degraded and assimilated. Assuming that extracellular enzymes are the only nitrogen containing by-products and the elemental formula of these enzymes can be written as $CH_{1.5}O_{0.31}N_{0.25}$ (Carlsen et al, 1996), the stoichiometric coefficient for extracellular enzymes (y_{enz}) was calculated. However, including this value into the carbon and degree of reduction balances we found that besides extracellular enzymes, still other by-products (y_{oth}) are formed. The stoichiometric coefficients (y_{oth}) and the degree of reduction of these other by-products (γ_{oth}) were calculated and are given in Table III. Since organic acids can be regarded as normal by-products of fungal fermentation and based on γ_{oth} we assume that *C. minitans* produces organic acids, such as citric acid (γ=3), fumaric acid (γ=3) or pyruvic acid (γ=3) on D2 and e.g. oxalic acid (γ=1) or formic acid (γ=2) on D1. However, no organic acids or other products were detected by HPLC-analysis. Since we are primarily interested in spore production, we did not investigate these by-products further.

From the data given in Table III, we can conclude that D2 is not a suitable medium for spore production since considerable amounts of by-products are produced and the stoichiometric coefficient for spores on starch is the lowest. D3 and D1 are equally efficient media for producing spores of *C. minitans* based on y_{sp}. These values for D1 and D3 given in Table III are comparable with the coefficients 0.035 and 0.317 for y_m and y_{sp} respectively found for *M. anisopliae* grown on rice husk (Dorta et al, 1996).

Kinetics of mycelial growth and spore production

The time courses of growth and sporulation of *C. minitans* on the three media are presented in Figures 1a-c. As can be seen, the profiles of mycelial growth, spore production and total biomass production follow the same pattern. Only on medium D1 a decrease in total amount of mycelium after the production phase (around day 10) is seen. Spore production starts approximately 2 days later than mycelial growth since first pycnidia, the mycelial structures that produce the spores, have to be formed. However, as can be seen from Figures 1a-c, after these two days both processes occur simultaneously. This is in contrast to *P. roqueforti* and *M. anisopliae* where first mycelium is produced before sporulation takes place and during the sporulation phase the amount of mycelium decreases (Desfarges et al, 1986; Dorta et al, 1996).

From the results in Figures 1a-c it appears that spore production is the highest on D3 (25 $g.l^{-1}$, $1.1*10^{12}$ $spores.l^{-1}$) which is expected based on previous experiments (Ooijkaas et al, 1998b and Ooijkaas et al, 1999). On D2 more spores are produced than on D1 on dry weight base (12 $g.l^{-1}$ vs. 5.8 $g.l^{-1}$); however, the spore numbers are almost equal ($4.3*10^{11}$ $spores.l^{-1}$ vs. $3.9*10^{11}$ $spores.l^{-1}$) which is in agreement with results from the past (Ooijkaas et al, 1998b). These data indicate that the spore weight differs on different media being $1.5*10^{-11}$ $g.spore^{-1}$, $2.9*10^{-11}$ $g.spore^{-1}$ and $2.5*10^{-11}$ $g.spore^{-1}$ respectively for D1, D2 and D3. Whether spore weight is related to quality (efficacy in the field) requires further study.

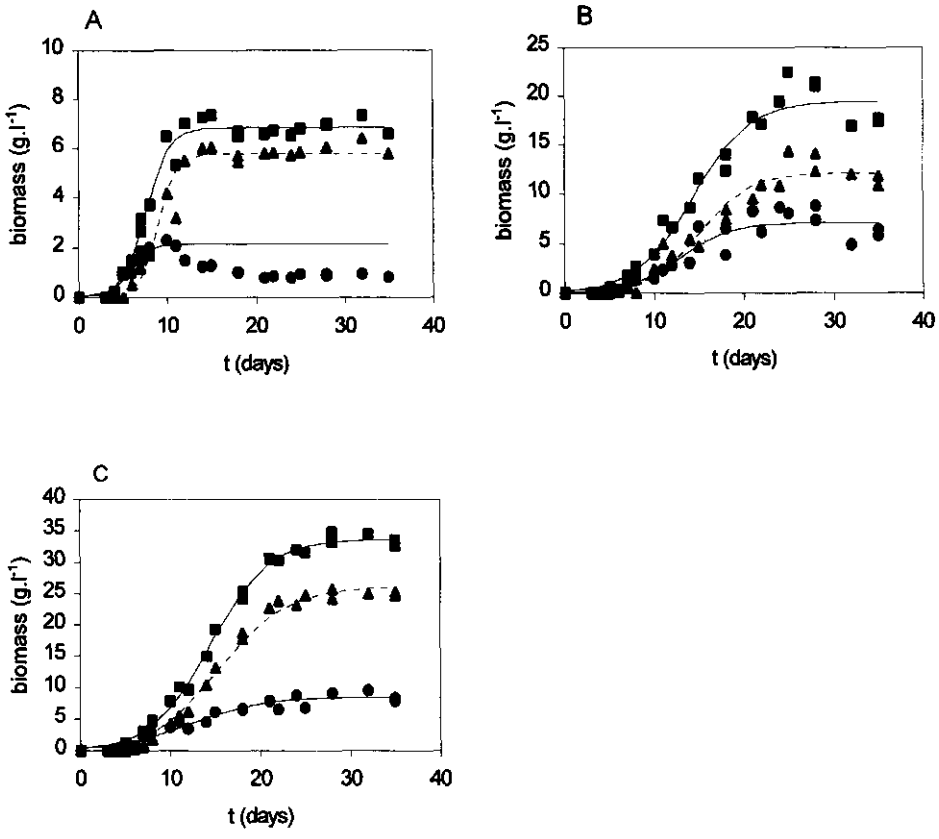

Figure 1: The experimentally determined total biomass (■), mycelium (●) and spore (▲) production at D1 (A), D2 (B) and D3 (C). The solid lines represents the logistic curves (eq. 6) and the dashed lines represents the spore production given by equation 10 fitted through the data.

The profiles of cumulative O_2, starch and urea consumption and CO_2 production (corrected for the amount of CO_2 released from urea) are given in Figures 2a-c. On medium D1, starch is completely consumed while for both D2 and D3 92% of the initial amount of starch is used. On medium D3, urea is completely used while 56% and 52% of the initial amount is consumed for D1 and D2 respectively. For all media, the O_2 consumption is almost equal to the CO_2 production resulting in a respiration quotient of about 1. Consequently, this

respiration quotient does not give any information on formation of by-products or the nature of these by-products. The total amount of CO_2 produced is 40% to 50% of the total amount of C-moles starch consumed for all three media.

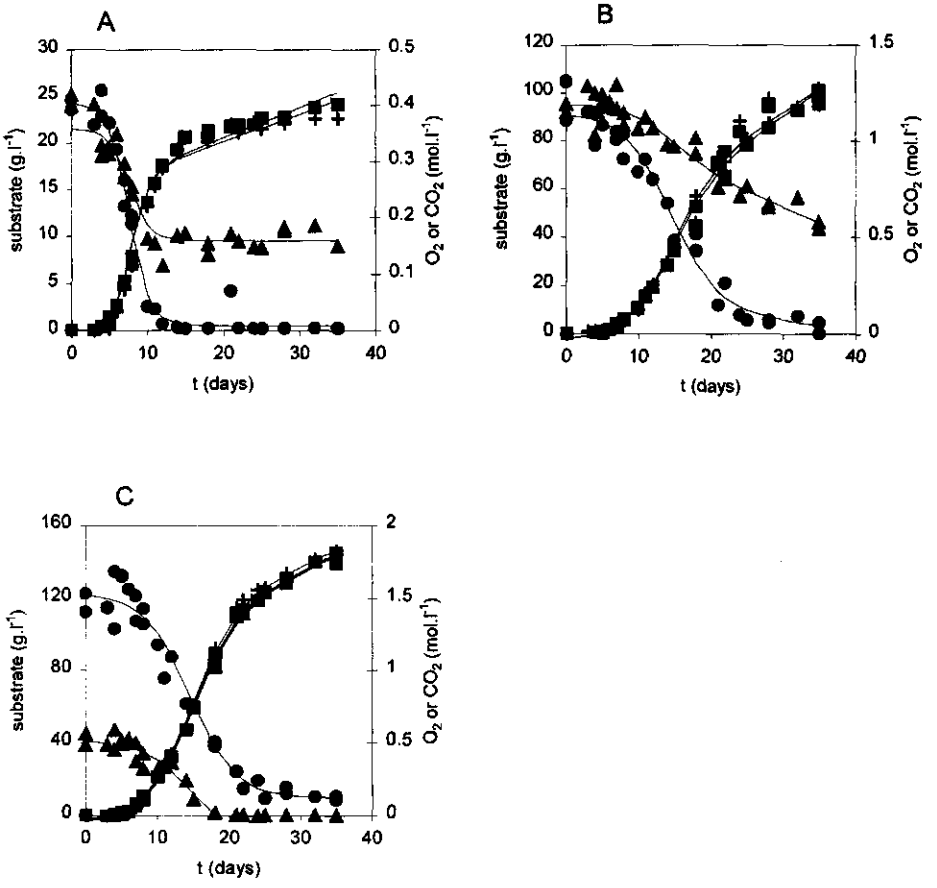

Figure 2: Profiles for starch (●), urea (▲) and cumulative O_2 (■) consumption and CO_2 (+) production at D1 (A), D2 (B) and D3 (C). The urea concentration is multiplied by 10. Solid lines are the fits of equation 8 through the points.

Modeling the kinetics of growth and sporulation of *C. minitans*

Frequently, fungal growth has been described by the logistic law (Koch, 1971; Smits et al, 1996; Sangsurasak et al, 1996):

$$\frac{dX}{dt} = \mu_{max} * \left(1 - \frac{X}{X_{max}}\right) * X \qquad (5)$$

Integration of eq. 5 gives:

$$X(t) = \frac{X_{max}}{\left(\frac{X_{max}}{X_0} - 1\right) * e^{-\mu_{max} * t} + 1} \qquad (6)$$

where X(t) is the actual amount of total biomass (mycelium and spores), X_{max} is the maximum attainable amount of biomass and μ_{max} is the maximum specific growth rate theoretically obtained for X=0. Although this empirical model does not give insight into what controls growth, it has been used to model the growth kinetics in many models of solid-state fermentation (Sangsurasak et al, 1996).

Generally, the substrate and oxygen consumption rates and carbon dioxide production rates can be described with the linear-growth model of Pirt (Smits et al, 1996; Roels, 1983):

$$\frac{dI}{dt} = Y_{ix} * \frac{dX}{dt} + m_i * X \qquad (7)$$

with Y_{ix} the true yield coefficient of compound i on biomass and m_{ix} the maintenance coefficient. In SSF this model is often used to correlate biomass production rate to carbon dioxide production rate or oxygen consumption rate (Smits et al, 1996; Okazaki et al, 1980; Ikasari and Mitchell, 1998). The usefulness of this correlation model is based on the relatively simple and rapid on-line measurements of carbon dioxide production and oxygen

consumption in most SSF processes, while direct biomass measurements are often very problematic.

Substitution of eq. 5 and 6 in eq. 7 and integration gives (eq.8):

$$I(t) = I_0 - X_{max} * \left[\frac{Y_{ix}}{\left(\frac{X_{max}}{X_0} - 1\right) * e^{-\mu_{max} * t} + 1} - \frac{Y_{ix}}{\left(\frac{X_{max}}{X_0}\right)} + \frac{m_i}{\mu_{max}} * Ln \left[\frac{\left(\frac{X_{max}}{X_0} - 1\right) * e^{-\mu_{max} * t} + 1}{\left(\frac{X_{max}}{X_0} - 1\right) * e^{-\mu_{max} * t}} \right] \right] \quad (8)$$

Eq. 8 describes the profiles for starch and urea consumption; to describe the profiles for cumulative O_2 consumption and CO_2 production the minus sign placed after I_0 was replaced by a plus sign. In eqs. 7 and 8 no separate term for by-products is included since the kinetics of by-products formation are unknown. In case of D2 where considerable amounts of by-products are formed, this will lead to an overestimation of Y_{ix}.

Eqs. 6 and 8 were fitted on the data from Figures 1a-c and 2a-c to estimate the parameters. The solid lines in Figures 1 and 2 Figures are the fitted curves. The estimates of the parameters are given in Table IV.

As can be seen from Figures 1and 2 and Table IV, these equations describe the total biomass production and substrate consumption or production patterns reasonably well.

From Table IV it appears that on D1 μ_{max} is the highest. However, this estimate of μ_{max} is related to that of X_0. In case of D2 and D3 the estimate of X_0 is unrealistically high, resulting in a relatively low estimate of μ_{max}. Therefore, no conclusions can be drawn on μ_{max}. Table IV shows that X_{max} increases, almost linearly, with increasing starch concentrations. More starch concentrations have to be tested to establish this relation between initial starch concentration and X_{max}.

There are almost no differences between D1 and D3 for both the yield and the maintenance coefficients. Surprisingly, for D1 and D3 m_s and m_n are about zero while m_o and m_e are larger than zero. This implies that by-products might be used for maintenance purposes on

D1 and D3. On D2 all maintenance coefficients are larger than on D1 and D3. This might be related to another metabolism caused by the high urea concentration or the relatively large amount of by-products formed on D2.

Table IV: Estimates for kinetic parameters ± the standard deviation obtained by fitting eq. 6 and 8 through the data in Figures 1 and 2.

			D1	D2	D3
Biomass	X_0	$g.l^{-1}$	0.023±0.013	0.208±0.100	0.362±0.064
	X_{max}	$g.l^{-1}$	6.84±0.11	19.46±0.61	33.68±0.39
	μ_{max}	day^{-1}	0.754±0.079	0.315±0.036	0.313±0.134
	r^2		0.984	0.971	0.996
starch	Y_{sx}	$g_s.g_x^{-1}$	3.485±0.136	4.116±0.335	3.538±0.260
	m_s	$g_s.g_x^{-1}.day^{-1}$	$1.82.10^{-4}$±0.006	0.020±0.022	$2.62.10^{-6}$±0.016
	r^2		0.984	0.970	0.968
urea	Y_{nx}	$g_n.g_x^{-1}$	0.174±0.017	0.122±0.026	0.122±0.014
	m_n	$g_n.g_x^{-1}.day^{-1}$	$2.09.10^{-6}$±0.001	0.006±0.002	$3.16.10^{-6}$±0.001
	r^2		0.920	0.922	0.939
O_2	Y_{ox}	$mol_o.g_x^{-1}$	0.044±0.002	0.040±0.002	0.041±0.001
	m_o	$mol_o.g_x^{-1}.day^{-1}$	$(7.65±0.79).10^{-4}$	$(1.30±0.14).10^{-3}$	$(6.89±0.48).10^{-4}$
	r^2		0.989	0.991	0.999
CO_2	Y_{cx}	$mol_c.g_x^{-1}$	0.046±0.002	0.042±0.002	0.043±0.001
	m_c	$mol_c.g_x^{-1}.day^{-1}$	$(6.78±0.90).10^{-4}$	$(1.20±0.15).10^{-3}$	$(6.37±0.57).10^{-4}$
	r^2		0.986	0.990	0.998

In the previous part mycelium and spore production were lumped together and treated as total biomass. However, it could be interesting to describe the profiles for mycelium production and spore production separately. Since the logistic curve is often used to describe biomass production in cases where no sporulation takes place, this curve (eq. 6) was fitted on the mycelium data of Figures 1 to estimate the parameters. For D1, the equation for mycelium production was fitted only till day 11, since the decline in mycelium is not described by this function. The dashed lines in Figure 1 are the fitted curves and the estimates of the parameters are given in Table V.

The kinetics of spore production are unknown. However, considering spores as a product of the mycelial biomass, spore production can be described with the linear-growth model of Pirt:

$$\frac{dSp}{dt} = Y_{spm} * \frac{dX}{dt} + m_{sp} * X \tag{9}$$

This equation, which distinguishes a growth-associated (Y_{spm}) and a non-growth-associated (m_{sp}) term was originally proposed by Luedeking and Piret for lactic acid fermentation (Roels, 1983). Based on the patterns in Figure 1, this equation was simplified by assuming that $m_{sp} = 0$. Since first pycnidia have to be produced before spores can be produced a lag-phase (λ) was introduced. These considerations lead to the following equation for spore production after substitution and integration:

$$Sp(t) = M_{max} * \left[\frac{Y_{spm}}{\left(\frac{M_{max}}{M_0} - 1\right) * e^{-\mu_{max}*(t-\lambda)} + 1} - \frac{Y_{spm}}{\left(\frac{M_{max}}{M_0}\right)} \right] \tag{10}$$

where Y_{spm} is the number of grams spores produced per gram mycelium produced, λ the lag-phase and M_{max} and M_0 the maximum attainable amount of mycelium and the amount of mycelium at t=0 respectively. Eq. 10 was fitted on the data from Figures 1a-c to obtain estimates of the parameters. The dashed lines in Figures 1 are the fitted curves. The estimates of the parameters are given in Table V.

As can be seen from Figure 1 and Table V, equations 6 and 10 describe mycelium and spore production reasonably well. Similar to total biomass production, μ_{max} for mycelium production seems to be the highest for D1. However, this is not certain owing to the link between M_0 and μ_{max}. Just as is the case for total biomass the estimate for M_0 is unlikely high for D2 and D3 and consequently, the estimates for μ_{max} are too low. As can be

concluded from Table V, Y_{spm} is clearly affected by medium composition. The assumption that sporulation is completely growth-associated (m_{sp}=0) seems to hold reasonably well.

Table V: Estimates of parameters ± the standard deviation obtained after fitting eq. 6 and 10 through the data in Figure 1

			D1	D2	D3
mycelium	M_0	$g.l^{-1}$	$(8.73\pm6.43)*10^{-3}$	0.070±0.068	0.363±0.124
	M_{max}	$g.l^{-1}$	2.15±0.10	7.14±0.39	8.47±0.33
	μ_{max}	day^{-1}	0.963±0.138	0.359±0.083	0.253±0.033
	r^2		0.951	0.898	0.952
spores	Y_{spm}	$g_{sp}.g_m^{-1}$	2.69±0.06	1.71±0.05	3.24±0.05
	λ	day	3.22±0.20	2.07±0.51	2.86±0.24
	r^2		0.973	0.960	0.993

Costs estimations

As shown by the outcome of the stoichiometry and kinetics, we might conclude that D3 is the optimal medium for spore production, since less substrate is spent for by-products and high amounts of spores are obtained. From Figures 1 and 2 it can be seen that to produce the same amount of spores obtained with one batch with D3 it is necessary to run three batches of D1. In the latter case less starch is needed than with D3 which thus might be more attractive if starch costs determine the production costs. In that case, the expensive Mes-buffer should be replaced by much cheaper alternatives.

To see which strategy is more attractive, the costs to produce the same amount of spores with each medium were roughly calculated. These first rough approximations of costs reveal that the production costs per ha for D1 and D3, given in Table VI, are more or less the same. As can be seen from this Table VI, the substrate costs are only a minor part of the production costs.

These cost calculations are based on the general model given by van 't Riet (1986), which only contains few microbial parameters and gross numbers for the production costs. In our costs calculations we take as estimate for the microbial parameters the data given in table V. Furthermore, we made the assumptions that fermentor costs are the same for solid-state fermentors and stirred liquid fermentors, that the production takes place in large-scale fermentors (> 100 m^3) and that an application dose consists of $5*10^{12}$ spores.ha^{-1} (Gerlagh et al, 1999). However, it is said that solid-state fermentation requires less energy and less capital investment than liquid fermentation (Barrios-González and Mejía, 1996), consequently the fermentor costs might be lower than calculated. In that case substrate costs will become more important. On the other hand, however, it is likely that production takes place in fermentors smaller than 100 m^3. The fermentor costs for small fermentors can rise to 10 $\$.m^{-3}.h^{-1}$ resulting in high production costs where substrate costs are just a minor part.

Table VI: Rough estimates of production costs based on assumptions mentioned in text

Substrate: $\$_{Starch} = 1\ \$.kg^{-1}$

fermentor: $\$_{hf} = 1\ \$.m^{-3}.h^{-1}$ (large-scale)

production costs: $\$_{pf} = 5*10^{12} * (\$_{ferm} + \$_{sub}) = 5*10^{12} * (\frac{\$_{hf} * t}{Sp_{tot}} + \frac{\$_{starch}}{Y^{ov}})$

	t (h)[1]	Sp_{tot} (#.m^{-3})[2]	Y^{ov} (spore.kg^{-1})[3]	$\$_{sub}$ ($.$ha^{-1}$)	$\$_{ferm}$ ($.$ha^{-1}$)	$\$_{pf}$ ($.$ha^{-1}$)
D1	275	$3.5*10^{14}$	$1.9*10^{13}$	0.3	3.9	4.2
D2	515	$3.8*10^{14}$	$4.2*10^{12}$	1.2	6.8	8.0
D3	635	$9.9*10^{14}$	$7.0*10^{12}$	0.7	3.2	3.9

[1] time needed to reach 90% of maximal spore production calculated with eq. 10

[2] 90% of maximum spore production, calculated as: $0.9*Y_{spm}*M_{max}$*spore weight; Y_{spm} and M_{max} are the estimates given in table V for the kinetic parameters of eq. 6 and 10 and spore weight being: $1.5*10^{-11}$ g.spore^{-1}, $2.9*10^{-11}$ g.spore^{-1} and $2.5*10^{-11}$ g.spore^{-1} for D1, D2 and D3 respectively

[3] Sp_{tot} divided by the initial starch concentration

In these calculations only the costs for starch and the fermentor are taken into account. However, the final costs will depend on more factors such as the costs for research,

downtime (i.e. cleaning, filling and sterilizing the fermentor), downstream processing, formulation and registration of *C. minitans* as biological control agent. It is expected that the costs for downtime and downstream processing will be higher for D1 and the other costs will be the same for D1 and D3. Therefore, the final production costs for D1 will most likely be higher than for D3, so D3 is the most efficient medium for spore production.

CONCLUSIONS

The stoichiometry of growth and sporulation was established by means of elemental balances. These balances also show that *C. minitans* produces by-products on all media. Simple empirical models could be used to describe the kinetics of growth, sporulation, carbon dioxide production and substrate consumption on all media. These models show that sporulation of *C. minitans* was growth-associated. Using these models, the process costs for producing spores were roughly calculated. It was shown that fermentor costs are the major part of the production costs.

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REFERENCES

Barrios-González, J. and Mejía, A. 1996. Production of secondary metabolites by solid-state fermentation. Biotechnol. Ann. Rev., 2, 85-121

Campbell, W.A. 1947. A new species of *Coniothyrium* parasitic on sclerotia. Mycologica, 39, 190-195.

Carlsen, M., Nielsen, J. and Villadsen, J. 1996. Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. J. Biotechnol., 45, 81-93.

Desfarges, C., Larroche, C. and Gros, J.B. 1987. Spore production of *Penicillium roquefortii* by solid-state fermentation: stoichiometry, growth and sporulation behavior. Biotechnol. Bioeng., 29, 1050-1058.

Dorta, B., Ertola, R.J. and Arcas, J. 1996. Characterization of growth and sporulation of *Metarhizium anisopliae* in solid-substrate fermentation. Enzyme Microbiol. Technol., 19, 434-439.

Erickson, L.E., Minkevich, I.G., and Eroshin, V.K. 1978. Application of mass and energy balance regularities in fermentation. Biotechnol. Bioeng., 20, 1595-1621.

Gerlagh, M., Goossen-van de Geijn, H.M., Fokkema, N.J. and Vereijken, P.F.G. 1999. Long-term biosanitation by application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-infected crops. Phytopathology, 89, 141-147.

Ikasari, L. and Mitchell, D.A. 1998. Oxygen kinetics during solid-state fermentation with *Rhizopus oligosporus*. Biotechnol. Techn., 12, 171-175

Koch, A.L. 1971. The kinetics of mycelial growth. J. Gen. Microbiol., 89, 209-216.

Larroche, C. and Gros, J.B. 1992. Characterization of the growth and sporulation behavior of *Penicillium roquefortii* in solid-substrate fermentation by material and bioenergetic balances. Biotechnol. Bioeng., 39, 815-817.

McQuilken, M.P., Budge, S.P. and Whipps, J.M. 1997a. Production, survival and evaluation of liquid culture-produced inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Biocontrol Sci. Technol., 7, 23-36.

McQuilken, M.P., Budge, S.P. and Whipps, J.M. 1997b. Effects of culture media and environmental conditions on conidial germination, pycnidial production and hyphal extension of *Coniothyrium minitans*. Mycol. Res., 101, 11-17.

McQuilken, M.P. and Whipps, J.M. 1995. Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol., 101, 101-110.

Okazaki, N., Sugama, S. and Tanaka, T. 1980. Mathematical model for surface culture of Koji mold. J. Ferment. Technol., 58, 471-476

Ooijkaas, L.P., Tramper, J. and Buitelaar, R.M. 1998a. Biomass estimation of *Coniothyrium minitans* in solid-state fermentation. Enzyme Microbiol. Technol., 22, 480-486.

Ooijkaas, L.P., Chin-Joe I., Tramper, J. and Buitelaar, R.M. 1998b. Spore production of *Coniothyrium minitans* during solid-state fermentation of different nitrogen sources with glucose or starch as carbon source. Biotechnol. Lett., 20, 785-788.

Ooijkaas, L.P., Wilkinson, E.C., Tramper, J. and, Buitelaar, R.M. 1999. Medium optimization of *Coniothyrium minitans* using statistically based experimental designs. Biotechnol. Bioeng., 64, 92-100.

Roels, J.A. 1983. Energetics and Kinetics in Biotechnology. Elsevier Biomedical Press, Amsterdam.

Sangsurasak, P., Nopharatana, M. and Mitchell, D.A. 1996. Mathematical modeling of the growth of filamentous fungi in solid-state fermentation. J. Sci. Ind. Res., 55, 333-342.

Smits, J.P., Rinzema, A., Tramper, J., Van Sonsbeek, H.M. and Knol, W. 1996. Solid-state fermentation of wheat bran by *Trichoderma reesei* QM9414: substrate composition changes, C-balance, enzyme production growth and kinetics. Appl. Microbiol. Biotechnol., 46, 489-496.

Weber, F.J., Tramper, J. and Rinzema, A. 1999. Quantitative recovery of fungal biomass grown on solid κ-carrageenan media. Biotechnol. Techn., 13, 55-58.

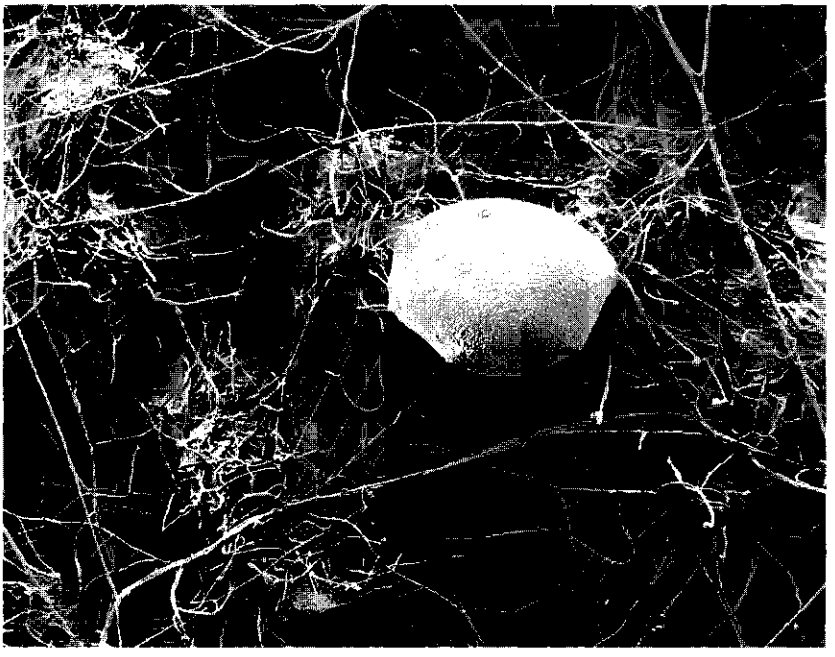
Whipps, J.M. and Gerlagh, M. 1992. Biology of *Coniothyrium minitans* and its potential for use in disease biocontrol. Mycol. Res., 96, 897-907.

Van 't Riet, K. 1986. The selection of research targets based on forecasted production costs. Trends Biotechnol., 4, 236-241.

CHAPTER 5

CHAPTER 6

DEFINED MEDIA AND INERT SUPPORTS: POTENTIAL AS SOLID-STATE FERMENTATION PRODUCTION SYSTEMS



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CHAPTER 6

ABSTRACT

Solid-state fermentation (SSF) using inert supports impregnated with (chemically defined) liquid media has potential in both scientific studies and industrial production of high-value products such as metabolites, biological control agents and enzymes. Due to the more defined system, SSF on inert supports offers favorable characteristics, such as better process control and monitoring and enhanced process consistency as compared to the cultivation on natural solid substrates such as grains or soybeans. Examples which illustrate the advantages of SSF on inert supports, comments on medium development and optimization, comments on support selection, and considerations regarding economics are presented in this paper.

INTRODUCTION

Solid-state fermentation (SSF) processes, involving the growth of microorganisms on moist solid substrate in the absence of free-flowing water, have considerable economical potential in producing products for the food, feed, pharmaceutical and agroindustry. However, due to the great success of large-scale submerged fermentation (SmF) processes, SSF was almost completely neglected in the Western World (Larroche and Gross, 1997). The last 15-20 years SSF has gained renewed interest in this part of the world, since it has certain advantages over SmF (Barrios-González and Mejía, 1996).

Two kinds of SSF systems can be distinguished depending on the nature of the solid phase used. The first and most commonly used and most often described system involves cultivation on a natural material that serves both as support and nutrient source. This system is referred to as cultivation on natural substrates. The second system involves the cultivation on an inert support impregnated with a liquid medium (Barrios-González and Mejía, 1996). Although the second system is not frequently used in SSF, it does exhibit favorable characteristics that are not observed with the cultivation on natural substrates.

This paper focuses on the application of SSF processes on inert supports impregnated with liquid (defined) media.

First the general features and advantages of SSF and then the production systems, especially the system using inert supports, are described. Furthermore, the development and optimization of suitable chemically defined liquid media is discussed, with special attention given to the use of statistically based experimental designs as alternative for the one-factor-at-a-time approach often used in optimization studies. In addition, considerations regarding the selection of a support in relation to reactor type are discussed in this paper and finally some future prospects including comments on economics are given. This is all illustrated with examples from literature clearly demonstrating the feasibility of SSF processes on inert supports impregnated with chemically defined media for both scientific and industrial purposes.

SOLID-STATE FERMENTATION

SSF is in fact a very old technology; it has been used extensively from ancient times in Asian and African countries. One of the oldest applications involves the making of bread by Egyptians 2600 BC (Larroche and Gross, 1997; Pandey 1992). Information on the "koji process" dates back to 1000 BC (Barrios-González and Mejía, 1996). Koji is an enzyme preparation produced by growing fungi such as *Aspergillus oryzae* on steamed rice or other cereals. It is still used as starter culture in the soy-sauce industry and in the fermentation of many Oriental foods. This koji process is of great historical importance to modern SSF technology and can be considered as the prototype of SSF.

It is evident that SSF exists for many centuries and various SSF processes with histories reaching far back in time are still practiced today. These processes deal with (Moo-Young et al, 1983): [1] fermented foods, e.g. tempeh, miso, pozol, [2] mold-ripened cheese, e.g. Roquefort, [3] starter cultures for fermented brews and [4] ensiling and composting. Recent applications of SSF include protein enrichment of agroindustrial residues, the production of

enzymes, organic acids and other fungal metabolites and spore production (Larroche and Gross, 1997).

As stated by several authors SSF could have advantages over SmF (Moo-Young et al, 1983; Barrios-González and Mejía, 1996). Amongst these advantages are improved product characteristics, higher product yields, easier product recovery, and reduced energy requirement. For instance, in SSF spore numbers of the biocontrol agent *Coniothyrium minitans* as high as 10^{15} spores.m^{-3} medium could be reached (Ooijkaas et al, 1999; Weber et al, 1999), while with SmF at most $6*10^{13}$ spores.m^{-3} medium could be obtained (McQuilcken et al, 1997). Furthermore, spores of the biocontrol agent *Trichoderma harzianum* produced by SSF showed both higher UV-resistance and longer viability after storage than those produced by SmF (Muñoz et al, 1995). In addition, spores of the mycoherbicide *Colletotrichum truncatum* produced by SSF retained higher viability after drying and storage and were more effective than spores produced by SmF (Silman et al, 1993).

Other examples, which clearly illustrate the benefits of SSF over SmF, are the 500-fold higher yield of chymosin in SSF as compared to SmF (Tsuchiya et al, 1994), the higher thermostability and pH tolerance of pectinases (Acuña-Arguelles et al, 1995), and the lower catabolite repression of enzyme production (Solís-Pereira et al, 1993).

PRODUCTION SYSTEMS

Two kinds of SSF systems can be distinguished depending on the nature of the solid phase used. The first and most commonly used system involves cultivation on natural materials that serve both as support and nutrient source. These materials are generally starchy or (ligno-)cellulosic agricultural products or agro-industrial sources such as grains and grain by-products, cassava, potato, beans, sugar beet pulp, etc. (Pandey, 1992). The second involves the cultivation on an inert support impregnated with a (chemically defined) liquid medium. Here, the solid support serves as anchorage point for the microorganism;

Table I: Examples of SSF using impregnated inert supports

Microorganism	Product	Support	Reference
Colletotrichum truncatum	Spores	Vermiculite, perlite	Silman et al, 1993; Silman et al, 1991
		Rice hulls	Silman et al, 1991
Beauveria bassiana	Spores	Clay granules	Desgranges et al, 1993
Penicillium roquefortii	Spores	Pozolano	Larroche et al, 1989
Coniothryium minitans	Spores	Hemp, bagasse, perlite	Weber et al, 1999
Gibberella fujikuroi	Gibberellic acid	Bagasse, PUF	Tomasini et al, 1997
Brevibacterium sp	L-Glutamic acid	Bagasse	Nampoothiri and Pandey, 1996
Aspergillus niger	Citric acid, polyols	Amberlite	Gutiérrez-Rojas et al, 1995
	Citric acid	Bagasse	Pintado et al, 1998
Penicillium chrysogenum	Penicillin	Bagasse	Barrios-González et al, 1988
Rhizopus delemar	Lipase	Amberlite	Christen et al, 1995
Penicillium citrinum	Nuclease P1	PUF	Zhu et el, 1994
Vibrio costicola	L-Glutaminase	Polystyrene	Nagendra Prabhu and Chandrasekaran, 1995
Aspergillus oryzae	Protease	PUF	Ozawa et al, 1996
	Amylase	PUF	Murado et al, 1997

materials proposed are hemp, perlite (Weber et al, 1999), polyurethane foam (PUF), sugarcane bagasse, vermiculite etc. (Larroche and Gross, 1992). Table I presents some examples of the wide application potential of SSF on inert supports regarding support, fungal species and products, but does not intend to be complete.

The natural substrates have as major disadvantage that the carbon source constitutes part of the structure. During growth of the microorganism the solid medium is degraded and as a result the geometric and physical characteristics of the medium change (Oriol et al, 1988b). Consequently heat and mass transfer can be reduced (Barrios-González and Meija, 1996). For example, oats shrinks due to the degradation of starch and evaporation of water and consequently channel formation can occur resulting in reduced mass and heat transfer (Weber et al, 1999). This disadvantage can be overcome by the use of an inert support with more or less constant physical structure throughout the process, allowing better control of heat and mass transfer.

The less complicated product recovery is an additional major advantage of SSF on inert supports over SSF on natural substrates. The extracellular products of interest can be easily extracted (e.g. by pressing) from the inert support. In this way, products can be obtained with fewer impurities as compared to the natural substrates and the support might be re-used. To illustrate this, spores of *P. roquefortii* formed inside buckwheat grains could only be recovered after disruption of the substrate while spores produced on pozolano particles (volcanic material) could be readily extracted without destroying the particles. Consequently, the recovery process is simplified and the particles are reusable (Larroche and Gross, 1989). Furthermore, the L-glutaminase extract produced by *Vibrio costicola* obtained from polystyrene was less viscous than that from wheat bran. In addition, the extract from polystyrene was free of undesired proteins while the wheat bran extract contained amylase and cellulase besides L-glutaminase (Nagendra Prabhu and Chandradekaran, 1995).

Other advantages of SSF on inert support over SSF on natural substrates are: it allows the design of adequate production media (Guttiérrez-Rojas et al, 1995) and mass balances for more advanced process modeling and process control are more easily established (Zhu et al,

1994), since all concentrations of the nutrients in the production medium are exactly known and can be analyzed at any time (Barrios-González and Meija, 1996)

DEVELOPMENT AND OPTIMIZATION OF THE PRODUCTION MEDIUM

Medium composition is a key parameter in optimizing SSF processes since nutritional factors such as carbon source, nitrogen, trace metals, etc. can all have an influence on growth and production of metabolites (Gutiérrez-Rojas et al, 1995), enzymes (Christen et al, 1995) and spores (Larroche, 1996). However, the possibilities of changing the medium composition and investigating the influence of certain medium components are limited with the natural substrates. Modification of the type and concentration is indirectly done by changing the grain, agricultural residue, etc., or by supplementation. The use of impregnated supports offers the possibility of flexibility in designing the medium for the optimal production of metabolites, enzymes or spores. In addition, the effects of certain medium components on the physiology, kinetics and production can be studied in detail. Moreover, process consistency is enhanced since the chemically defined medium inherently supports a more reproducible process while on the contrary due to the heterogeneity or batch-to-batch variation of the natural medium the process performance may vary.

Initial development of a chemically defined medium for SSF may start with a more or less arbitrary selection of a carbon source, nitrogen source, inorganic salts, trace elements and growth factors. This arbitrarily chosen medium might already give satisfactory results. Mostly, however, after initial formulation of the medium the appropriate nutrients and concentrations that will support the best production should be established. Usually, this is done by varying one factor while keeping the other factors at a constant level. However, this one-factor-at-a-time approach, although simple, often requires a considerable amount of work and time, especially when more factors have to be studied. In addition, if the effect of one factor is dependent on the level of another factor then this information will not be obtained by the one-factor-at-a-time approach. Nevertheless, this method is often used to

study the influence of components and to optimize the chemically defined medium. For example, the influence of several carbon and nitrogen sources on spore production of *C. minitans* (Ooijkaas et al, 1998), *Epicoccum nigrum* (Ting Zhou et al, 1996) and *Talaromyces flavus* (Engelkes et al, 1997) is studied using the one-factor-at-a-time technique.

Screening and optimization of nutrients can be done in a more efficient way by using statistically designed experiments, which has become popular especially in industry. These designs, which involve simultaneously varying several factors in a planned manner, are useful in identifying the important nutrients and interactions between two or more nutrients with a minimum of testing. This approach generally starts with screening which medium components have a significant effect on the desired response. Fractional factorial designs, one class of experimental designs, can be used for screening which medium components in an already formulated medium have a significant effect and should be further optimized (Zhu et al, 1996; Ooijkaas et al, 1999). However, Plackett-Burman designs are more efficient for screening purposes if on forehand it is not known which components should be present in the medium (Srinivas et al, 1994; Ramana Murthy et al, 1999). Further optimization of these variables can then be done by for instance central composite designs and response surface analysis. In this way, the optimal combination of these variables that give the best response is found in an effective manner.

As an example, a Plackett-Burman design was used to select the best nutrients amongst 23 nutrients for cyclosporin A production. Central composite design and response surface analysis were then used to optimize the concentrations of the 5 medium components obtained from the Plackett-Burman design (Ramana Murthy et al, 1999). Furthermore, a chemically defined medium for spore production of *C. minitans* was optimized by a factor 7 using experimental designs. In the first step the influence of 7 medium components on spore production was evaluated using fractional factorial design. In the second step the concentrations of the three nutrients that significantly affect spore production were further optimized using central composite design and response surface analysis (Ooijkaas et al, 1999).

CHAPTER 6

CHOICE OF SUPPORT MATERIAL

Evaporation is a very effective mechanism to remove the metabolic heat produced during cultivation in larger-scale reactors for SSF (Gutiérrez-Rojas et al, 1996). However, evaporation can affect the water activity (a_w) and might therefore have an adverse effect on the activity of the fungus (Weber et al, 1999; Larroche et al, 1992; Oriol et al, 1988a). In addition, evaporation can also cause shrinkage leading to channeling in the substrate bed and consequently poor process control (Weber et al, 1999). Replenishment of the water lost would therefore be desirable. In non-mixed systems this is virtually impossible, as strong moisture gradients can not be prevented. Therefore, just as with natural supports, inert supports should ideally be able to release a large amount of water without affecting the a_w (Weber et al, 1999). Weber et al. proposed a strategy to evaluate the use of various supports in a packed-bed reactor. Based on literature and laboratory data in combination with heat and mass balances, a rational selection of a support can be made. This procedure is schematically depicted in Fig. 1.

First, allowable values for the inlet and outlet temperatures are chosen. Ideally this choice is based on knowledge of the dependence of the microbial activity on temperature. Secondly, the microbial heat production is estimated from the O_2 consumption rate of a microorganism growing on the solid substrate measured in isothermal lab experiments. Next, the aeration rate that will give an increase in temperature from T_{in} to T_{out} is calculated by means of a simplified enthalpy balance. When this aeration rate is feasible, it is checked whether the loss of water will affect the cultivation. In order to evaluate this, the required cultivation time, the sorption isotherm of the support and the effect of a_w on the microorganism should be known.

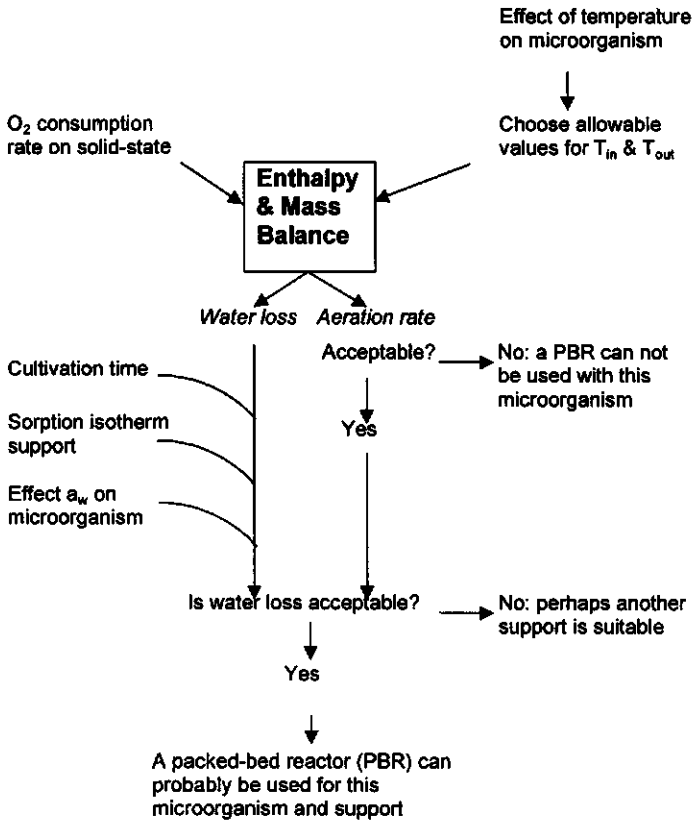

Figure 1: Schematic decision procedure for selecting an inert support and reactor

This approach was used to decide which of the support materials hemp, bagasse or perlite could be used in large-scale packed-bed reactors for spore production of *C. minitans* (Weber et al, 1999). It was shown that moisture control is the limiting factor for cultivation in a packed-bed reactor. Of the three inert supports tested, hemp provides the best spore yield, control of water activity due to its high water uptake capacity and the fact that it does not shrink upon evaporation.

Agitation of the medium promotes mass and heat transfer (Lonsane et al, 1992; Stuart et al, 1999). Furthermore, mixing offers the possibility of homogeneous water addition, allowing compensation of water losses caused by evaporation. Consequently, supports with less

absorption capacities can be used in mixed reactors. In mixed reactors other support characteristics should be taken into account in selecting the support. The support should not form agglomerates since aeration and mixing will become more difficult when the support particles stick together (Larroche and Gros, 1989). Moreover, the support should retain its structure and withstand the shear force caused by the agitation (Stuart et al, 1999).

Besides the support, the fungus should be able to withstand the shear forces as well. On some supports the activity of fungi is adversely affected by the mixing, whereas on other supports no adverse effects of mixing were observed (Stuart et al, 1999). It is anticipated that on some supports the fungus is better protected against the damaging shear forces than on others, resulting in a higher productivity.

In conclusion, an arbitrary choice of support might still give satisfactory production on laboratory scale, but on larger scale the same support could give poor production. The choice of support depends on the type of reactor used. In non-mixed reactors heat and mass transfer problems could occur or growth could be limited by low water content when an inappropriate support is used. In mixed reactors the support should withstand the shear force caused by mixing and give protection of the mycelium against agitation.

ECONOMICS

Realization of SSF on impregnated inert supports as a commercial system depends on its feasibility. Aspects to consider include medium costs, fermentor costs, costs of downstream processing and formulation, registration costs and product value. The higher costs of the defined media make natural media the preferred choice, especially for low-cost products. The latter system makes use of agricultural materials that usually are inexpensive and widely available. However, for high-added-value products such as certain metabolites, specific enzymes or biologically active products, SSF on inert supports can be used since the medium costs are usually a fraction of the overall production costs. For example, preliminary costs calculations for spore production of *C. minitans* on chemically defined

media have shown that the fermentation accounts for more than 80 % and substrate costs account for less than 20% of the production costs (Ooijkaas et al, submitted). In these calculations only the costs for the defined medium and the fermentation are taken into account. Therefore, if downstream processing, formulation and registration are considered as well, the medium costs will become even less important.

It is known that downstream processing can markedly affect the overall production costs due to the recovery losses. This situation can be improved by improving the recovery yield and decreasing the number of steps in the downstream processing (Van 't Riet, 1986). Since downstream processing is simplified and improved with inert supports as compared to natural substrates (Larroche and Gross, 1989; Nagendra Prabhu and Chandradekaran, 1995) it is anticipated that this will affect the overall production costs in a positive way and consequently outweigh the higher medium costs.

CONCLUSIONS AND PERSPECTIVES

Inert supports impregnated with defined media will continue to be a valuable tool in research, and will become more important for some industrial processes due to the favorable characteristics that are not observed with natural substrates. SSF on inert supports impregnated with defined media facilitates reproducible and detailed physiological and kinetic studies in SSF, which will eventually be the basis for efficient process development, control strategies and reactor design. Furthermore, SSF on impregnated inert supports offers the potential for better process control and monitoring due to the more defined environment. In addition, similar to laboratory studies, this system supports a more reproducible fermentation process, an important and desired characteristic for any industrial fermentation process. However, in the end economy will be the decisive factor in determining the success of SSF on inert supports. The advantages will at least for some processes outweigh the higher medium costs as compared to SSF on natural substrates.

Therefore, it can be expected that the use of impregnated inert supports as SSF production system will become more attractive, especially for high-added-value products.

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REFERENCES

Acuña-Argüelles, M.E., Guttiérrez-Rojas, M. Viniegra-González, G. and Favela-Torres, E. 1995. Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation. Appl. Microbiol. Biotechnol., 43, 808-814.

Barrios-González, J. and Mejía, A. 1996. Production of secondary metabolites by solid-state fermentation. Biotechnol. Ann. Rev., 2, 85-121.

Barrios-González, J., Tomasini, A., Viniegra-González, G. and López, I. 1988. Penicillin production by solid-state fermentation. Biotechnol. Lett., 10, 793-798.

Christen, P., Angeles, N., Corzo, G., Farres, A. and Revah, S. 1995. Microbial lipase production on a polymeric resin. Biotechnol. Techn., 9, 597-600.

Desgranges, C., Vergoignan, C., Lereec, A., Riba, G., and Durand, A. 1993. Use of solid-state fermentation to produce *Beauveria bassiana* for the biological control of European corn borer. Biotechnol. Adv., 11, 577-587.

Engelkes, C.A., Nuclo, R.L. and Fravel, D.R. 1997. Effect of carbon, nitrogen, and C:N ratio on growth, sporulation and biocontrol efficacy of *Talaromyces flavus*. Phytopathol., 87, 500-505

Gutiérrez-Rojas, M., Amar Aboul Hosn, S., Auria, R., Revah, S. and Favela-Torres, E. 1996. Heat transfer in citric acid production by solid-state fermentation. Process Biochem., 31, 363-369.

Guttiérrez-Rojas, M., Córdova, J., Auria, R., Revah, S. and Favela-Torres, E. 1995. Citric acid and polyol production by *Aspergillus niger* at high glucose concentration in solid-state fermentation on inert support. Biotechnol. Lett., 17, 219-224.

Larroche, C. 1996. Microbial growth and sporulation behaviour in solid-state fermentation. J. Sci. Ind. Res., 55, 408-423.

Larroche, C. and Gros, J.B. 1989. Strategies for spore production by *Penicillium roquefortii* using solid-state fermentation techniques. Process Biochem. 24, 97-103.

Larroche, C. and Gros, J.B. 1992. Characterization of the behavior of *Penicillium roquefortii* in solid-state cultivation on support by material balances. J. Ferment. Bioeng., 74, 305-311.

Larroche, C. and Gros, J.B. 1997. Special transformation processes using fungal spores and immobilized cells. Adv. Biochem. Eng/Biotechnol. 55, 179-220.

Lonsane, B.K., Saucedo-Castaneda, G., Raimbault, M., Roussos, S., Viniegra-Gonzalez, G., Ghildyal, N.P., Ramakrishna, M. and Krishnaiah, M.M. 1992. Scale-up strategies for solid-state fermentation systems. Process Biochem., 27, 259-273.

McQuilken, M.P., Budge, S.P. and Whipps, J.M. 1997. Production, survival and evaluation of liquid culture-produced inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Biocontro. Sci. Technol., 7, 23-36.

Moo-Young, M. Moreira, A.R. and Tengerdy, R.P. 1983. Principles of solid-state fermentation. In: The filamentous fungi, vol. 4: Fungal Technology. Smith, J.E., Berry, D.R. and Kristiansen, B. [eds]. Edwin Arnold, London, p117-144.

Muñoz, G.A., Agosin, E. Cotoras, M., San Martin, R. and Volpe, D. 1995. Comparison of aerial and submerged spore properties for *Trichoderma harzianum*. FEMS Microbiol. Lett., 125, 63-70.

Murado, M.A., González, M.P., Torrado, A. and Pastrana, L.M. 1997. Amylase production of *Aspergillus oryzae* on polyurethane foams. Some mechanistic approaches from an empirical model. Process Biochem., 32, 35-42.

Nagendra Prabhu, G. and Chandrasekaran, M. 1995. Polystyrene-an inert carrier for L-glutaminase production by marine *Vibrio costicola* under solid-state fermentation. World J. Microbiol. Biotechnol. 11, 683-684.

Nampoothiri, K.M. and Pandey, A. 1996. Solid-state fermentation for L-glutamic acid production using *Brevibacterium* sp. Biotechnol. Lett., 18, 199-204

Ooijkaas, L.P., Chin-Joe, I., Tramper, J. and Buitelaar, R.M. 1998. Spore production of *Coniothyrium minitans* during solid-state fermentation on different nitrogen sources with glucose or starch as carbon source. Biotechnol. Lett., 20, 480-486.

Ooijkaas, L.P., Buitelaar, R.M., Tramper, J. and Rinzema, A. Growth and sporulation stoichiometry and kinetics of *Coniothyrium minitans* in solid-state fermentation. Submitted.

Ooijkaas, L.P., Wilkinson, E.C., Tramper, J. and Buitelaar, R.M. 1999. Medium optimization for spore production of *Coniothyrium minitans* using statistically based experimental designs. Biotechnol. Bioeng., 64, 92-100.

Oriol, E., Raimbault, M., Roussos, S. and Viniegra-Gonzales, G. 1988a. Water and water activity in the solid-state fermentation of cassava starch by *Aspergillus niger*. Appl. Microbiol. Biotechnol. 27, 498-503.

Oriol, E., Schettino, B., Viniegra-Gonzales, G. and Raimbault, M. 1988b. Solid-state culture of *Aspergillus niger* on support. J. Ferment. Technol., 66, 57-62.

Ozawa, S., Sato, K. and Endo, I. 1996. Repeated batch production of alkaline protease by solid-state fermentation using urethane foam as carriers. Bioprocess. Eng., 14, 63-68.

Pandey, A. 1992. Recent process developments in solid-state fermentation. Process Biochem. 27, 109-117.

Pintado, J., Lonsane, B.K., Gaime-Perraud, I. and Roussos, S. 1998. On-line monitoring of citric acid production in solid-state culture by respirometry. Process Biochem., 33, 513-518.

Ramana Murthy, M.V., Mohan, E.V.S. and Sadhukhan, A.K. 1999. Cyclosporin-A production by *Tolypocladium inflatum* using solid-state fermentation. Process Biochem 34, 269-280.

Silman, R.W., Bothast, R.J. and Schisler, D.A. 1993. Production of *Colletotrichum truncatum* for use as a mycoherbicide: effects of culture drying and storage on recovery and efficacy. Biotech. Adv., 11, 561-575.

Silman, R.W., Nelsen, T.C. and Bothast, R.J. 1991. Comparison of culture methods for production of *Colletotrichum truncatum* spores for use as a mycoherbicide. FEMS Microbiol. Lett., 79, 69-74.

Solís-Pereira, S., Favela-Torres, E., Viniegra-González, G. and Guttiérrez-Rojas. 1993. Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid-state fermentations. Appl. Microbiol. Biotechnol., 39, 36-41.

Srinivas, M.R.S., Chand, N, and Lonsane, B.K. 1994. Use of Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid-state fermentation. Bioprocess Eng., 10, 139-144.

Stuart, D.M., Mitchell, D.A., Johns, M.R. and Litster, J.D. 1999. Solid-state fermentation in rotating drum bioreactors: operating variables affect performance through their effects on transport phenomena. Biotechnol. Bioeng., 63, 383-391.

Tomasini, A., Fajardo, C. and Barrios-González, J. 1997. Gibberellic acid production using different solid-state fermentation systems. World J. Microbiol. Biotechnol., 13, 203-206.

Tsuchiya, K., Nagashima, T., Yamamoto, Y., Gomi, K., Kitamoto, K., Kumagai, C. and Tamura, G. 1994. High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. Biosci. Biotech. Biochem., 58, 895-899.

Van 't Riet, K. 1986. The selection of research targets based on forecasted production costs. Trends Biotechnol., 4, 236-241.

Weber, F.J., Tramper, J. and Rinzema, A. 1999. A simplified material and energy balance approach for process development and scale-up of *Coniothyrium minitans* conidia

production by solid-state cultivation in a packed-bed reactor. Biotechnol. Bioeng., 65, 447-458.

Ting Zhou, Reeleeder, R.D. and Sparace, S.A. 1996. Influence of nutrients on growth of *Epicoccum nigrum*. Can. J. Microbiol., 42, 647-654.

Zhu, Y., Smits, J.P., Knol, P. and Bol, J. 1994. A novel solid-state fermentation system using polyurethane foam as carrier. Biotechnol. Lett., 16, 643-648.

Zhu, Y., Knol, W., Smits, J.P. and Bol, J. 1996. Medium optimization for nuclease P1 production by *Penicillium citrinum* in solid-state fermentation using polyurethane foam as carrier. Enzyme Microbiol. Technol., 18, 108-112.

SUMMARY

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During recent years, the use of fungal spores for the biological control of plant pests and diseases has received increasing interest. Mass production of spores is an essential step to commercialize these biological control agents; hence, reliable mass production methods are needed. Solid-state fermentation (SSF) is theoretically the preferred production method since most fungi sporulate well on solid substrates. In addition, SSF often produces biocontrol agents of better quality than liquid fermentation. However, rational design and operation of an SSF-process for production of fungal conidia are hampered by several factors. One of them is the lack of knowledge about the physiology and the kinetics of fungal growth and sporulation in SSF.

In this thesis, research on the physiology and kinetics of growth and sporulation in SSF of the biological control agent *C. minitans* is described. The quantity of biomass is an essential variable in physiological and kinetic studies. However, direct measurement of biomass is almost impossible in SSF since fungi penetrate into and bind tightly to the solid substrate. Therefore, various indirect methods to estimate the amount of biomass, being respiration measurements and several biochemical analyses, were evaluated for *C. minitans*. As discussed in Chapter 2, none of the tested indicators are perfect for biomass estimations of *C. minitans* on all media tested. However, following the CO_2 production and/or O_2 consumption (respiration) during the cultivation period gives valuable online information about the progress of the growth and the spore production process. Of the biochemical analyses tested only the amount of protein or nucleic acids can be used to estimate roughly the total amount of biomass. Though, these analyses were not useful for all media tested. Therefore, it can be concluded that we did not find a universally applicable indirect method to estimate the amount of biomass of *C. minitans* in SSF.

In Chapter 2 it can also be read that medium composition affects growth and sporulation, however, the influence of medium components on sporulation of *C. minitans* is poorly understood. Therefore, the influence of the main medium components, carbon and nitrogen, was studied in more detail using chemically defined media, which facilitate reproducible studies. Several nitrogen sources in combination with glucose or starch were evaluated for their influence on sporulation of *C. minitans* (Chapter 3). Since not only source but also concentration and C/N-ratio can affect sporulation, a low and high C-concentration were tested while keeping the C/N-ratio constant by likewise changing the N-concentration. It

was shown that urea and glycine are the preferred nitrogen sources since high concentrations were not inhibiting sporulation and the pH remained stable during cultivation. However, urea was chosen for further studies since glycine might be used as carbon source as well by the fungus. Furthermore, it was concluded that starch was preferred to glucose since at high starch concentrations spore numbers were all a factor two or more higher than at high glucose concentration. Therefore, starch in combination with urea as nitrogen source was used for further studies.

This chemically defined medium was further optimized with respect to spore quantity (Chapter 4) using statistically based experimental designs. These designs are more efficient than varying one factor at time. In the first optimization step the influence of starch, urea, phosphate, magnesium, calcium, thiamin and trace elements on spore production was evaluated using a fractional factorial design. Starch and trace elements influenced spore production positively while urea affected spore production negatively. The other components had no significant influence on spore production. In the second and third steps the concentrations of starch, urea and trace elements were further optimized using central composite designs and response surface analysis. This optimization strategy allowed the spore production to be increased by a factor 7 from $4*10^9$ to almost $3*10^{10}$ spores per Petri dish of 9-cm diameter corresponding with $2*10^8$ to $3*10^{12}$ spores.kg^{-1} medium.

For the optimal design and control of a solid-state fermentation process, mathematical models can be useful tools. These models require quantitative estimates of growth and sporulation of *C. minitans* on solid substrates and information on the stoichiometry of the bioconversion reactions. The stoichiometry and kinetics of mycelium and spore production were studied (Chapter 5) on defined media with different concentrations of starch, urea and trace elements (the low and high concentrations of Chapter 3 and the optimal concentrations of Chapter 4). By means of elemental balances the stoichiometry of growth and sporulation was established. It was concluded that *C. minitans* produces by-products on all media, especially in the medium with high urea concentrations where 30% of the starch is converted into by-products.

Simple empirical models were used to describe the kinetics of growth, sporulation, carbon-dioxide production and substrate consumption on these three media. Total biomass and mycelium could be described reasonably well with the logistic law. Starch, urea and

oxygen consumption, and carbon-dioxide production could be described as function of total biomass by the linear-growth model of Pirt. Similar to substrate consumption and carbon-dioxide production, the kinetics of sporulation could be described as function of mycelium production with the linear-growth model. Based on the kinetics, the process costs for producing spores were roughly calculated. It was shown that fermentor costs form the major part of the production costs.

In all these laboratory studies the use of a chemically defined medium was very useful. It facilitates reproducible and detailed physiological and kinetic studies in SSF, which will eventually be the basis for efficient process development, control strategies and reactor design. The use of an inert carrier impregnated with a chemically defined medium, as alternative for agricultural substrates in SSF, might be attractive in industrial processes too. Due to the more defined system, SSF on inert carriers offers favorable characteristics, such as better process control and monitoring and enhanced process consistency as compared to the cultivation on natural solid substrates. Examples, which illustrate the advantages of using inert carriers impregnated with defined media, together with comments on medium development and optimization, support selection and economics are given in Chapter 6. It was concluded that the advantages will at least for some processes outweigh the higher medium costs as compared to SSF on natural solid substrates. This is especially true for high-added-value products, such as biologically active products, where medium costs are usually a fraction of the total production costs, as is the case for *C. minitans*. Based on the considerations given in Chapter 6, it is expected that the use of inert carriers impregnated with defined media as SSF production system will have industrial potential for *C. minitans* and other (high-added-value) products.

SAMENVATTING

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Gedurende de laatste jaren is er steeds meer interesse gekomen voor biologische bestrijdingsmiddelen gebaseerd op schimmelsporen. Om deze middelen commercieel aantrekkelijk te maken zijn betrouwbare grootschalige produktiesystemen nodig. Theoretisch is vaste-stof fermentatie oftewel solid-state fermentation (SSF) de meest geschikte methode om schimmelsporen te produceren aangezien de meeste schimmels goed sporuleren op vaste substraten. Ook is het zo dat sporen geproduceerd met SSF vaak van betere kwaliteit zijn dan wanneer deze biologische bestrijdingsmiddelen met vloeistof fermentatie geproduceerd zijn. Echter het op een rationele wijze ontwerpen en bedrijven van een SSF-proces om schimmelsporen te produceren wordt bemoeilijkt door verschillende factoren. Een ervan is het gebrek aan kennis op het gebied van fysiologie en kinetiek van schimmelgroei en sporulatie op vaste substraten.

In dit proefschrift wordt het onderzoek naar de fysiologie en kinetiek van *Coniothyrium minitans*, een biologisch bestrijdingsmiddel, in SSF beschreven. In fysiologische en kinetische studies is de hoeveelheid biomassa een belangrijke variabele. Echter directe meting van de biomassa is nagenoeg onmogelijk in SSF aangezien schimmels het vaste substraat binnendringen en zich daar stevig kunnen hechten. Daarom zijn verschillende indirecte methoden om de hoeveelheid biomassa te schatten getest (hoofdstuk 2), te weten respiratie metingen en verschillende biochemische analyses. Het bleek echter dat geen van deze indirecte metingen een perfecte schatter is voor de hoeveelheid biomassa van *C. minitans* op elk getest medium. Niettemin gaf het volgen van de CO_2-produktie of O_2-consumptie (respiratie) waardevolle on-line informatie over het verloop van het groei- en sporulatieproces. Van de geteste biochemische analyses kunnen alleen het eiwitgehalte en het nucleinezuurgehalte gebruikt worden om de totale hoeveelheid biomassa ruw te schatten. Echter deze analyses waren niet bruikbaar als schatter voor de hoeveelheid biomasa voor alle geteste media. Daarom is geconcludeerd dat er geen algemeen toepasbare indirecte methode is om de hoeveelheid biomassa van *C. minitans* te schatten in SSF.

In hoofdstuk 2 is ook te lezen dat het gebruikte medium invloed heeft op groei en sporulatie van *C. minitans*; niettemin is nauwelijks bekend hoe mediumsamenstelling sporulatie beïnvloedt. De invloed van de belangrijkste mediumcomponenten, koolstof en stikstof, op sporulatie is nader onderzocht waarbij gebruik gemaakt is van een chemisch gedefinieerd medium wat reproduceerbare studies vergemakkelijkt. In hoofdstuk 3 is de invloed van

verschillende stikstofbronnen in combinatie met zetmeel of glucose op sporulatie beschreven. Aangezien niet alleen bron maar ook concentratie en C/N verhouding een invloed kan hebben, zijn twee C-concentraties getest, een lage en een hoge concentratie, terwijl de C/N-verhouding constant gehouden werd door gelijktijdig ook de N-concentratie te veranderen.

Aangetoond is dat ureum en glycine de meest geschikte stikstofbronnen zijn aangezien hoge concentraties geen negatief effect op sporulatie hebben en de pH tijdens kweken stabiel bleef. Daar glycine mogelijk ook als koolstofbron gebruikt kan worden is gekozen voor ureum in verder onderzoek. Bovendien is geconcludeerd dat zetmeel te prefereren is boven glucose aangezien sporen aantallen een factor twee of meer hoger zijn bij hoge zetmeel concentraties dan bij hoge glucose concentraties. In verdere studies is daarom gebruik gemaakt van zetmeel als koolstofbron gecombineerd met ureum als stikstofbron.

Dit verkregen chemische gedefinieerd medium is verder geoptimaliseerd op sporen aantallen m.b.v. op statistiek gebaseerde proefopzetten. Deze proefopzetten zijn efficiënter dan het variëren van telkens één variabele. In de eerste optimalisatiestap is gebruik gemaakt van een gefractioneerde factoriele proefopzet waarmee de invloed van zetmeel, ureum, fosfaat, magnesium, calcium, thiamine en sporenelementen bepaald is. Zetmeel, ureum en sporenelementen bleken een invloed te hebben op sporen produktie, terwijl de andere componenten geen significante invloed hadden op de sporulatie. In de tweede en derde stap zijn de concentraties zetmeel, ureum en sporenelementen verder geoptimaliseerd door gebruik te maken van "central composite designs" en "response surface analysis". Deze optimalisatie strategie resulteerde in een verhoging van de sporenproduktie met een factor 7 van $4*10^9$ tot bijna $3*10^{10}$ sporen per petrischaal. Omgerekend komen deze getallen overeen met aantallen van $2*10^8$ tot $3*10^{12}$ sporen per kg medium.

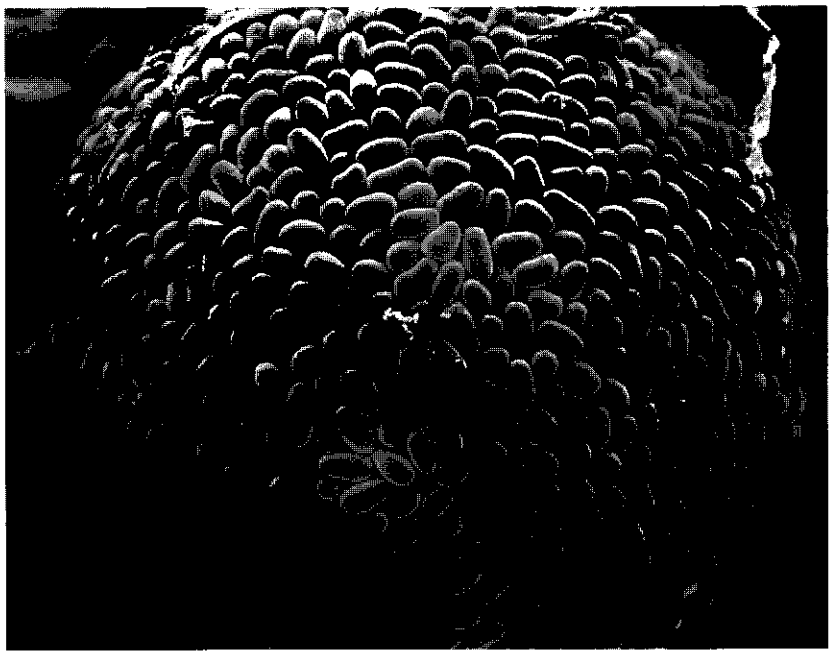
Bij het optimaal ontwerpen en beheersen van een SSF-proces kunnen wiskundige modellen handige hulpmiddelen zijn. Deze modellen hebben goede kwantitatieve schattingen nodig van groei en sporulatie naast informatie over de stochiometrie van de omzettingsreacties. De stoichiometrie en kinetiek van mycelium en sporenproduktie is bestudeerd op gedefinieerd medium met verschillende concentraties zetmeel, ureum en sporenelementen. (de lage en hoge concentraties uit hoofdstuk 3 en de optimale concentraties zoals gegeven in hoofdstuk 4). De stoichiometrie van groei en sporulatie is bepaald met behulp van

elementbalansen. De conclusie is dat *C. minitans* op elk medium (ongewenste) bijprodukten maakt, vooral in het medium met de hoge ureum en zetmeel concentraties waarbij 30% van het aanwezige zetmeel omgezet is in bijprodukten.

De kinetiek van groei, sporulatie, koolstofdioxideproduktie en substraatconsumptie is beschreven aan de hand van eenvoudige empirische modellen. De hoeveelheid mycelium en hoeveelheid totale biomassa kunnen behoorlijk goed beschreven worden met het logistische groeimodel. Zetmeel-, ureum-, zuurstofconsumptie en koolstofdioxideproduktie kunnen beschreven worden als functie van de hoeveelheid totale biomassa met het lineaire-groeimodel van Pirt. Analoog aan substraatconsumptie en koolstofdioxideproduktie kon het lineaire-groeimodel gebruikt worden om de sporenproduktie als functie van de hoeveelheid mycelium te beschrijven. Gebaseerd op deze modellen zijn de produktiekosten om sporen te produceren grof berekend. Hiermee is aangetoond dat de fermentorkosten het grootste deel uitmaken van de totale kosten.

In al deze studies op labschaal was het gebruik van een chemisch gedefinieerd medium erg handig. Het vergemakkelijkte reproduceerbare en gedetailleerde fysiologische en kinetische studies welke de basis zijn voor een efficiënte procesontwikkeling, controle strategieën en reactorontwerp. Het gebruik van een inerte drager geïmpregneerd met een chemisch gedefinieerd medium als alternatief voor natuurlijke vaste substraten kan ook aantrekkelijk zijn voor industriële processen. Doordat SSF op inerte dragers een meer gedefinieerd systeem is, biedt het voordelen t.o.v. SSF op natuurlijke substraten waaronder betere procescontrole en -bewaking en constantere processen. Voorbeelden die de voordelen van inerte dragers geïmpregneerd met gedefinieerd medium illustreren worden gegeven in hoofdstuk 6, samen met commentaar aangaande ontwikkeling en optimalisatie van het medium, dragerselectie en kosten. Daar wordt ook aangetoond dat voor verschillende processen de voordelen ruimschoots op wegen tegen de hogere mediumkosten t.o.v. natuurlijke substraten. Dit geldt vooral voor hoogwaardige produkten, zoals biologische bestrijdingsmiddelen, waar mediumkosten maar een klein deel uitmaken van de totale produktiekosten, zoals het geval is voor *C. minitans*. De verwachting is, gebaseerd op de overwegingen gegeven in hoofdstuk 6, dat het gebruik van inerte dragers geïmpregneerd met gedefinieerd medium als SSF-produktiesysteem industriële potentie zal hebben voor de sporenproduktie van *C. minitans* en andere (hoogwaardige) produkten.

SAMENVATTING IN GEWONE MENSENTAAL



Door Wimjan de Moes

SAMENVATTING IN GEWONE MENSENTAAL

Onbegrepen zwoegen zij voort; hun beste jaren verglijden in sombere laboratoria waar de ene na de andere proef de mist ingaat; is het geen muis-arm, dan kwellen hen rechthoekige monitor ogen. We hebben het over AiO's, OiO's en verwante mensachtigen. Jaren kan het duren voor zij eindelijk resultaat van hun slavenarbeid aanschouwen: een proefschrift of andere publicaties. (Triangel, Studenten Pastoraat, jaargang 21, juli 1999)

Tot de in dit citaat genoemde mensachtigen behoort ook Lydia. Het proefschrift wat zij als eindresultaat heeft geproduceerd staat vol onbegrijpelijke taal, die wij, gewone mensen, waarschijnlijk niet helemaal kunnen volgen. Om hier enige duidelijkheid in aan te brengen voor ons de gewone mensen, heb ik geprobeerd om in begrijpelijk Nederlands uit te leggen wat Lydia zoal in de afgelopen 4 jaar heeft uitgespookt.

In de 1947 heeft ene meneer Campbell een schimmel ontdekt die een andere schimmel die bij planten rot veroorzaakt opat. Het was dus mogelijk deze schimmel als biologisch bestrijdingsmiddel te gebruiken. In die jaren was dit echter niet interessant want er waren goedkopere en 'betere' chemische bestrijdingsmiddelen. Tegenwoordig is die interesse er wel dus is breder onderzoek noodzakelijk.

De door meneer Campbell ontdekte schimmel heet *Coniothyrium minitans*. Ik zal haar Coni noemen, dit spreekt wat makkelijker. Coni is dus een schimmel die als bestrijdingsmiddel gebruikt kan worden. Op kleinere schaal, in veldproeven, is gebleken dat dit biologisch bestrijdingsmiddel goed werkt. Verder is Coni niet schadelijk voor mens en dier omdat zij bij ca. 38 ° C niet kan groeien.

Om Coni commercieel aantrekkelijk te maken moet er veel van geproduceerd worden. Coni moet dus zo snel mogelijk voor een grote familie gaan zorgen, en daarbij zo effectief mogelijk te werk te gaan.

Het beginstadium van Coni is een bolletje (zie foto blz. 115). Als Coni groeit gaat zij draden maken, deze draden vertakken zich, en dit proces gaat door tot er een wirwar van draden met her en der dikkere kluwens van is ontstaan. Voor Lydia zijn deze draden en kluwens niet zo interessant. Maar op een gegeven moment gaat Coni zich vermenigvuldigen en ontstaan er in de kluwens druppels met miljoenen bolletjes, allemaal kleine Coni's. En dit is wel interessant voor Lydia. Deze kleine Coni's kunnen namelijk gebruikt worden als biologisch bestrijdingsmiddel of om nog meer Coni's te kweken.

Natuurlijk moet er zo nu en dan gekeken worden of Coni wel goed groeit en een grote familie wordt. Dit bleek zeer lastig. De draden en bolletjes van Coni gaan door de hele voedselbron heen zitten, en waren niet direct te meten of te wegen. Daarom heeft Lydia een aantal indirecte methoden gebruikt; zie hiervoor hoofdstuk 2. Ikzelf kan hier wel dieper op ingaan, maar het bleek dat geen van deze indirecte methoden geschikt was om de groei van Coni te meten. Uit dit deel van het onderzoek kwam ook de uitspraak voort "geen resultaat is ook resultaat".

Omdat er geen goede indirecte methode was heeft Lydia, weliswaar via een omweg, toch een directe methode gebruikt in haar proefschrift. Hiertoe heeft zij de familie Coni en het overgebleven voedsel in een maatbeker gestopt, waarna het geheel met een soort staafmixer werd geblend. Wat er overblijft is een slurrie van kapotte draden, voedsel en bolletjes. Door deze bolletjes te tellen kan Lydia zien of Coni een grote familie geworden is.

Om Coni te laten groeien en vermenigvuldigen moet zij wel goed te eten krijgen. Lydia heeft verschillende voedselbronnen voor Coni uitgeprobeerd; in hoofdstuk 3 en 4 (voor wie gedetailleerde informatie wil) is dit duidelijk omschreven. Het komt er eigenlijk op neer dat Coni het liefst houdt van een combinatie van zetmeel (b.v. aardappels), ureum (b.v. vlees), en sporen elementen (b.v. diverse groenten) in de juiste balans. Dus als men zo naar de eetgewoontes van Coni kijkt, groeit zij net als ons het best op een uitgekiend dieet. Coni is dus helemaal geen raar klein beestje, maar lijkt een beetje op ons.

Lydia heeft verder ook geprobeerd dit hele proces van groeien en vermenigvuldigen in enkele wiskundige modellen te stoppen. Deze wiskundige modellen - voor de liefhebber zie hoofdstuk 5 - kunnen o.a. gebruikt worden door technologen om een industrieel proces te ontwikkelen. Ook kunnen met deze modellen de globale productiekosten geschat worden; voor de commerciëlen onder ons, dit heeft Lydia al gedaan in hoofdstuk 5. In hoofdstuk 6 kletst zij nog wat over alternatieve voedselbronnen. Zij heeft in haar proefschrift kunstmatige voedselbronnen gebruikt, en volgens Lydia is dat niet alleen handig voor onderzoek, maar ook voor een industrieel proces.

Dit is nu in gewone mensentaal uitgelegd het onderwerp waar Lydia zich de afgelopen 4 jaar mee bezig heeft gehouden. Samengevat in pak 'm beet 3 uurtjes.

CURRICULUM VITAE

Lydia Priscilla Ooijkaas werd 24 juli 1970 te Nijverdal geboren. Na behalen van haar VWO-diploma aan het College Noetsele te Nijverdal in 1988, begon zij datzelfde jaar met haar studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit Wageningen (LUW), nu Wageningen Universiteit geheten. Zij koos Levensmiddelenbiotechnologie als specialisatie waarvoor afstudeervakken gevolgd werden bij de sectie Industriële Microbiologie en de sectie Proceskunde (beide LUW), en een stageperiode doorgebracht werd bij DSM-Research te Geleen. In maart 1994 studeerde zij af, waarna zij als practicum assistent aan de slag ging bij de sectie Proceskunde. In april 1995 begon zij als onderzoeker in opleiding (OiO) bij het Instituut voor Agrotechnologisch Onderzoek (ATO) met haar promotieonderzoek. De daar behaalde resultaten staan beschreven in dit proefschrift. Sinds juni 1999 is Lydia werkzaam als postdoc onderzoeker bij de sectie Proceskunde.

BIJSCHRIFT BIJ DE FOTO'S:

Blz 1 en 115 Detailopname druppel gevuld met sporen (bolletjes) van *Coniothyrium minitans.*

Blz 9 Zijaanzicht van een pycnide, het vruchtlichaampje waarin de sporen van *C. minitans* gevormd worden.

Blz 31 Bovenaanzicht mycelium (draden) en pycniden (kluwens) van *C. minitans* na 5 dagen kweken.

Blz 41 Detailopname bovenaanzicht mycelium en pycniden van *C. minitans* na 5 dagen kweken.

Blz 65 Bovenaanzicht mycelium en pycniden van *C. minitans* na 10 dagen kweken. Uit enkele pycniden verschijnen druppels gevuld met sporen.

Blz 89 Een pycnide waaruit een druppel gevuld met sporen komt.

Blz 107 Detailopname van een druppel gevuld met sporen uit een pycnide.